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PRINCIPAL INVESTIGATOR: David A. Gewirtz, Ph.D.

CONTRACTING ORGANIZATION: Virginia Commonwealth University
Richmond, Virginia 23298-0568

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David A. Gewirtz, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Virginia Commonwealth University
Richmond, Virginia 23298-0568

E-MAIL:
gewirtz@hsc.vcu.edu

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13. ABSTRACT ***(Maximum 200 Words)***

We have determined that neither p53 status nor alterations in levels of the Myc protein are critical factors in radiosensitivity or in sensitivity to adriamycin. The refractoriness of breast tumor cells to DNA damage induced apoptosis may be related, in part, to upregulation of p21 as well as to stimulation of MAP kinase activity. Our current studies using cells carrying a p21 antisense vector should clarify the role of p21 while studies relating to the MAP kinase pathway are being initiated. Pretreatment of p53 wild type breast tumor cells with Vitamin D3 compounds sensitizes the cells to ionizing radiation and to adriamycin, in part through the promotion of apoptosis - suggesting that the Vitamin D compounds can be used to enhance the effectiveness of radiotherapy and chemotherapy in the clinical treatment of breast cancer. Finally, we are attempting to determine how p53 status influences the fidelity of double-strand break repair in apoptosis-proficient 184B5 breast epithelial cells, and the possible relation between apoptosis and tolerance for misrepair. Such studies may suggest additional candidates for transgenic manipulation of the response to radiation.

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FOREWORD

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INTRODUCTION *Subject and scope of research* This work is directed towards developing an understanding of the molecular and signal-transduction events mediating growth arrest and cell death in breast tumor cells after exposure to ionizing radiation. Our studies have been focused primarily on the p53, $p21^{waf1/cip1}$, Myc and E2F-1 proteins; these proteins have overlapping and possibly mutually exclusive functions in the regulation of cell growth and apoptotic cell death pathways in response to DNA damage. Our findings that the breast tumor cell fails to undergo apoptotic cell death in response to irradiation (as well as in response to adriamycin) have provided the incentive for developing approaches for radiosensitization (and chemosensitization) of the breast tumor cell. In addition, we have discovered that irradiation has the capacity to promote the uptake and expression of exogenous genes, a finding which is the basis for the development of strategies for the delivery of cytotoxic and apoptosis-promoting genes to both p53 wild-type and p53 mutated breast tumor cells. Finally, the possible role of p53 in enforcing the fidelity of double-strand break repair is being investigated in matched p53+ and p53-defective breast epithelial cells. Putative double-strand break misrepair events, induced by bleomycin and detected as *HPRT* mutations, are being analyzed at both the chromosomal level and the DNA sequence level. Chromosomal stability, delayed reproductive death, and radiation-induced apoptosis and cell cycle perturbations are being assessed in the mutant cells in an attempt to determine whether specific types of misrepair events were accompanied by changes in these responses.

Background While radiation therapy and chemotherapy using the drug adriamycin are effectively utilized in the management of breast cancer, the recurrence of disease indicates the limitations of these treatment protocols. We believe that breast tumor cells may demonstrate primary resistance to radiotherapy (and to chemotherapy), in part through their refractoriness to the induction of apoptotic cell death. Furthermore, even in breast tumor cells which are initially responsive to radiotherapy and chemotherapy, the absence of apoptotic cell death may permit the acquisition of a radioresistant and chemoresistant phenotypes during the course of treatment.

An extensive literature describes the closely-linked signal transduction pathways which mediate growth arrest and/or cell death in cells which incur DNA damage by irradiation or. Drugs such as adriamycin Irradiation, as well as other modalities which induce DNA damage are known to up-regulate levels of the tumor suppressor protein, p53 (Kuerbitz et al, 1992; Zhan et al, 1993; Dulic et al, 1994; Gudas et al, 1995), which in turn increases levels of the cyclin-dependent kinase inhibitory protein, $p21^{waf1/cip1}$ (Di Leonardo et al, 1994; Dulic et al, 1994; Bae et al, 1995; Gudas et al, 1995). Inhibition of cyclin dependent kinases results in abrogation of the phosphorylation of the tumor suppressor protein, Rb (Nigg et al, 1995; Dimri et al, 1996) - which then binds to and inactivates the transcription factor, E2F (Chellappan et al, 1991; Hiebert et al, 1992; Almasan et al, 1995). E2F is thought to regulate the expression of a spectrum of genes associated with DNA synthesis including c-*myc*, DNA polymerase alpha, thymidine kinase and thymidine synthetase (Almasan et al, 1995; Martin et al 1995). Interference with E2F function is postulated to block DNA synthesis and promote growth arrest (Johnson et al, 1993; Almasan et al, 1995).

While the p53, Myc and E2F proteins are fundamental components of the G1 cell cycle checkpoint, all of these proteins have also been shown to mediate apoptosis or programmed cell death

in a variety of tumor cell models in response to DNA damage (Evan et al, 1992; Almasan et al, 1995; Henneking et al, 1995; Lowe et al, 1995). Conversely, up regulation of $p21^{waf1/cip1}$ in response to DNA damage is though to abrogate the apoptotic pathway (Lin and Benchimol, 1995: Attardi et al, 1996). Although many types of DNA damage can cause an increase in p53 levels and activate the cascade described above, this pathway appears to be particularly sensitive to double strand breaks. Indeed, transfection experiments have suggested that the presence of one double-strand break in a cell nucleus, even on a nonessential plasmid, can activate a p53-dependent checkpoint and arrest the cell in G1 (Huang et al, 1996). In addition, the enhanced apoptotic responses of cells with defective double-strand break repair suggest that double-strand breaks may be the critical triggering lesion for radiation-induced apoptosis as well (Meng et al., 1998; Nussenzweig et al., 1997). Thus, the upstream events in radiation-induced G1 arrest and apoptotic cell death may be intim ately linked to the recognition and processing of double-strand breaks. Moreover, the possible implication of the *BRCA1* and *BRCA2* (hereditary breast cancer) gene products in double-strand break repair, by virtue of their association with the known repair factor hRad51 in nuclear foci in irradiated cells (Bishop et al., 1998; Chen et al., 1999), may suggest a specific link between double-strand break repair and breast cancer.

Purpose The goal of these studies has been to understand the role of c-*myc* and the p53 protein in the pathway leading to growth arrest in the breast tumor cell. As indicated in the body of this report, we have made significant progress relating to this component of the proposal. In the course of this work, we have concluded that the relative refractoriness of breast tumor cells to the induction of apoptotic cell death in response to radiation or chemotherapeutic agents which induce DNA damage represents an observation with potentially important clinical ramifications. Consequently, we have extended our efforts to develop approaches for the promotion of apoptotic cell death in both p53 wild-type and p53 mutated breast tumor cells. An additional component of this work was to investigate the repair of free radical-mediated double-strand break (using bleomycin as a model radiomimetic agent) in breast epithelial cells having wild-type versus mutant p53 genes, and the possible relationship of double-strand break repair and repair fidelity to cell death.

BODY

Four of the six specific aims composing this grant relate to the suppression of c-myc expression and Myc protein levels in the response of the breast tumor cells to radiation and the linkage of alterations in c-myc expression and function to upstream regulators of the DNA damage response pathway (p53 and p21). In order to evaluate the general applicability of the data generated with ionizing radiation, we have extended this work to one of the primary drugs utilized in the treatment of breast cancer, the anthracycline antibiotic, adriamycin. We have established that the breast tumor cell is relatively refractory to apoptosis (programmed cell death) in response to both radiation and adriamycin. Consequently, we have further modified the direction of our research to develop approaches for sensitizing the breast tumor cell to adriamycin and radiation through the promotion of apoptotic cell death.

In the fifth specific aim, as revised in the previous annual report, we proposed to compare the

frequency and molecular nature of both small deletions and gene rearrangements induced by bleomycin in 184B5 (p53+) and 184B5-E6c6 (p53-) cells. In the sixth specific aim (also revised), we proposed to determine whether gene rearrangements in the two cell lines are accompanied by (1) translocations specifically involving the X chromosome, (2) global chromosomal instability, (3) changes in radiation-induced cell cycle perturbations, (4) apoptosis and (5) delayed reproductive death.

A. Involvement of p53 and Myc in radiosensitivity, chemosensitivity, growth arrest and cell death in response to irradiation and adriamycin.

Utilizing two p53 wild-type (MCF-7 and ZR-75) and two p53 mutated (MDA-MB231 and T-47D) breast tumor cell lines, we have substantiated the absence of apoptotic cell death in response to ionizing radiation and/or the chemotherapeutic agent, adriamycin (Fornari et al, 1996; Magnet and Gewirtz ; Di and Gewirtz; Jones and Gewirtz). The data in the manuscript by Magnet and Gewirtz indicates that ionizing radiation produces prolonged growth arrest in ZR-75-1 and MCF-7 breast tumor cells and further supports the concept that these irradiated cells fail to undergo apoptosis (based on cell morphology, cell-cycle analysis and the lack of DNA fragmentation). Figure 1 indicates that as reported for the p53 wild-type MCF-7 breast tumor cells (Fornari et al, 1996) adriamycin initially produces a small degree of cell death (loss of approximately 20% of the cell population) in the p53 mutated MDA-MB231 and T-47D breast tumor cells; however, this is non-apoptotic cell death which is followed by prolonged growth arrest (Fornari et al, 1996; Di and Gewirtz; Jones and Gewirtz) .

While suppression of c-*myc* expression and Myc protein levels in growth arrest appears to hold for a variety of DNA damaging agents in MCF-7 breast tumor cells, this does not appear to be a universal component of the DNA damage response pathway as irradiation fails to suppress Myc levels in ZR-75 cells (Magnet and Gewirtz) as shown in Figure 2.

Figure 3 indicates that sensitivity to adriamycin was identical in the p53 wild-type MCF-7 and the p53 mutated MDA-MB231 breast tumor cell line. We can conclude from these as well as the preceding studies (Watson et al, 1997) that neither p53 status nor alterations in levels of the Myc protein are critical factors in either radiosensitivity or chemosensitivity, at least in those cases where the tumor cell fails to undergo apoptotic cell death.

B. Relationship of the DNA damage response pathway involving p53 and $p21^{waf1/cip1}$ to the refractoriness of the breast tumor cell to apoptosis in response to irradiation and adriamycin.

It has been reported that increases in levels of $p21^{waf1/cip1}$ are antagonistic to the apoptotic pathway (Lin and Benchimol, 1995; Attardi et al, 1996). We find that radiation as well as adriamycin produce profound increased in $p21^{waf1/cip1}$ levels in the p53 wild-type cells (Magnet and Gewirtz, Di and Gewirtz). In order to test this hypothesis, we have been working to develop a breast tumor cell line

expressing p21 which will be used to assess the effects of dysregulation of the cyclin-dependent kinase inhibitor p21 on the response of breast cancer cells to ionizing radiation and to adriamcyin.

C. Studies to develop a breast tumor cell line expressing p21 antisense to assess the effects of dysregulation of the cyclin-dependent kinase inhibitor p21 on the response of breast cancer cells to ionizing radiation.

Attempts to stably transfect MCF-7 cells with a p21 antisense construct, as we had previously done in the case of both HL-60 and U937 human leukemia cells (Freemerman et al) , proved unexpectedly difficult. Three successive attempts at transfection of cells with the construct, limiting dilution to obtain single cell clones, and screening of the resulting clones for an impaired p21 response to IR, were unsuccessful, in that all of the clones exhibited p21 induction equal to that of empty vector controls. In each of these attempts, 20-30 individual clones were screened.

As an alternative approach, we obtained an adenoviral expression vector from Dr. Paul Fisher, Columbia College of Physicians and Surgeons, and, with the assistance of Dr. Kristoffer Valerie, Director of the Core Adenovirus Laboratory at MCV, attempted to transfect MCF-7 and ZR-745 cells with these vectors. As seen in the accompanying figures, attenuation of the p21 response to 5 Gy of IR was not attenuated even at MOI values as high as 500.

Finally, on our fourth attempt, we were able to stably transfect the MCF-7 line with our p21 antisense construct. As shown in Figure 4, two clones (AS1/IR and AS2/IR) were obtained which exhibited an 80% and 95% reductions respectively in p21 induction compared to empty vector controls following an equivalent 6-hr exposure to 5 Gy. These lines are now being subcloned in an attempt to obtain sublines which display an even greater attenuation of p21 induction in response to IR. Once these are obtained, the proposed studies examining the impact of p21 induction on IR-mediated cell death and antiproliferative effects in breast cancer cells will proceed, hopefully within the next 1-2 months.

D. Radiosensitization and chemosensitization of the breast tumor cell; promotion of apoptosis by exposure of cells to Vitamin D3 and the hypocalcemic Vitamin D3 analogs EB 1089 and RO-23-7553.

We have determined that Vitamin D3 and the Vitamin D3 analogs EB 1089 and RO-23-7553 induce growth arrest alone but fail to promote apoptotic cell death in the MCF-7 breast tumor cell line (Sundaram and Gewirtz; Chaudhry, Sundaram and Gewirtz). Similarly, ionizing radiation and adriamycin fails to promote apoptotic cell death (Fornari et al, 1996; Watson et al, 1997; Magnet and Gewirtz; Di and Gewirtz; Jones and Gewirtz). However, the combination of either EB 1089 with ionizing radiation (Sundaram and Gewirtz) and the combination of either EB 1089 or RO-23-7553 with adriamycin resulted in an enhanced antiproliferative effects (Figure 5 and Chaudhry et al) accompanied by the promotion of apoptosis. These effects appear to be limited to p53 wild-type cells, although this is a tentative conclusion that is currently being explored.

These studies demonstrate that pretreatment of p53 wild type breast tumor cells with Vitamin D3 compounds sensitizes the cells to ionizing radiation as well as to adriamycin. Sensitization appears

to occur through the promotion of apoptosis and not through an increase in the extent of initial DNA damage. This leads to the tantalizing possibility that the Vitamin D analogs can be used to enhance the effectiveness of radiotherapy and chemotherapy in the clinical treatment of breast cancer.

E. Utilization of ionizing radiation to promote gene uptake and apoptotic cell death in p53 mutated breast tumor cells.

The Vitamin D compounds promote apoptosis in response to irradiation in p53 wild-type breast tumor cells; however a similar effect was not evident in p53 mutated MDA-MB231 cells or T-47 D cells (Sundaram and Gewirtz). Consequently, since many breast cancers present with mutated p53 genes, we have been interested in developing approaches for inducing cell death in p53 mutated cells.

Ionizing radiation enhanced the liposome-mediated delivery and expression of the SV-40 luciferase transgene in MDA-MB231 breast tumor cells (Jain and Gewirtz). Improved transgene delivery and expression was observed at a clinically relevant dose of 2 Gy and was dose-dependent over a range of 2-10 Gy in both MCF-7 and MDA-MB231 breast tumor cells. Furthermore, enhancement of gene uptake was observed with irradiation prior to, coincident with and after transfection. These findings indicate that irradiation can enhance the efficiency of liposomal mediated transgene uptake. We propose that irradiation could be combined with gene therapy in the treatment of breast cancer. In this context, we have demonstrated that pharmacological concentrations of estradiol can enhance the uptake, nuclear translocation and expression of p53 in p53 mutated MDA-MB231 breast tumor cells, with the induction of apoptosis (Jain and Gewirtz).

F. Mutagenesis

Experimental Methods Medium used for routine culture of 184B5 cells (hereafter referred to as serum-containing medium) was a 1:1 mixture of Ham's F12 and Dulbecco's media, reconstituted from powder (Gibco), filter-sterilized, and supplemented with 0.5 μg/ml hydrocortisone, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 25 μg/ml insulin, 5% horse serum, and antibiotics. Procedures requiring more stringently controlled growth, or cloning of individual cells, were performed using commercially prepared Mammary Epithelial Cell Growth Medium (MEGM) from Clonetics Corp. This medium contains, in addition to the usual low-molecular-weight species, bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone and antibiotics, but no serum.

Cells were grown in 100-mm plastic Petri dishes, and were routinely subcultured when they reached 80% confluence. For subculturing, cells were washed with PBS and detached with 0.25% trypsin in PBS containing 1 mM EDTA. An equal volume of serum-containing medium was added, and the cells were pelleted (100 × *G*, 10 min) in order to remove trypsin. The cells were resuspended and seeded at a density of 10^5 per dish in the serum-containing medium.

For mutagenesis experiments, each 100-mm plate was seeded with 8×10^5 cells in serum-containing medium. The next day, the medium was replaced with identical medium but containing hypoxanthine, amethopterin and thymidine (HAT), in order to eliminate any preexisting *HPRT*

mutants. When the cells reached 80% confluence, the medium was replaced with MEGM medium without HAT and lacking epidermal growth factor. Beginning 48 hr later, these G_0 cells were treated with bleomycin (0.5 - 5 μg/ml) for two days, with both medium and drug being replaced after one day. Following the 2-day treatment, the drug was removed, the cells washed, and the medium again replaced with drug-free, growth factor-free MEGM. After 4 hr of recovery in this medium, the cells were trypsinized and an aliquot was plated at 800 cells per plate in complete MEGM medium to determine survival. The bulk of the cells were seeded at 10^5 cells per plate in the serum-containing medium, with 10 plates for each treated culture. The cells usually reached confluence about 12 days later, and at that time they were again trypsinized and plated in MEGM medium containing 10 μM 6-thioguanine (5×10^4 cells per plate, two plates from each subculture, 20 plates from each initial treated culture) in order to select mutants. After 12 days, colonies were counted and two mutant colonies from each initial culture were trypsinized inside a cloning ring. The trypsinized cells were seeded into 100-mm plates with serum-containing medium. The cells were subcultured once and then trypsinized, washed, and frozen in medium containing 10% DMSO prior to storage in liquid nitrogen.

For molecular analysis of *HPRT* mutations, each mutant clone was expanded to ~5×10^6 cells, and whole-cell RNA was isolated using an RNA-STAT/60 kit (Tel-Test). Following cDNA synthesis with Moloney murine leukemia virus reverse transcriptase, the *HPRT* message was amplified by two-step PCR using nested *HPRT* primers (McGregor et al., 1991), and then sequenced using several primers from within the coding sequence. A RETROscript kit (Ambion) was used for cDNA synthesis and amplification, and an Epicentre Cycle sequencing kit was used for DNA sequencing. The RNA from each line was also subjected to Northern blot analysis as described above but using a probe consisting of bases 333-999 of the *HPRT* cDNA.

For cytogenetic analysis, 184B5 cells (Walen and Stampfer, 1989), its E6-transfected derivative 184B5-E6c6 (Gudas et al., 1995), and various bleomycin-induced *HPRT* mutants of these lines, were grown to 80% confluence and incubated for 4 hr in the presence of Colcemid to accumulate mitotic cells. The cells were swollen by a 10-min exposure to 0.075 M KCl, fixed with 3:1 methanol/acetic acid, and dropped onto cold, wet slides. The slides were hybridized for 2 days at 37°C in a humidified atmosphere with the SkyPaint mixture of fluorescent probes (Applied Spectral Imaging), which stains each human chromosome a different color. Karyotype analysis was performed using a Leica DMRBE microscope with CCD camera, and SpCube 2.0 software.

Results and Discussion

Bleomycin-induced mutagenesis in matched p53+ and p53- mammary epithelial cell lines As described in the previous annual report, we had already shown that the 184B5 human mammary epithelial cell line (p53+) retained normal radiation-induced G1 arrest, while the HPV E6-transfected derivative 184B5-E6c6, previously shown to lack p53 (Gudas et al., 1995), also lacked any detectable G1 arrest. Furthermore, we had shown that bleomycin was significantly mutagenic in both cell lines, and that the background frequency of spontaneous mutants could be decreased by incubation of the cells in medium containing hypoxanthine, aminopterin and thymidine (HAT). Aminopterin blocks *de novo* nucleotide synthesis pathways, so that only cells with intact *HPRT* genes (and thus able to make

purine nucleotides from hypoxanthine through the salvage pathway) can survive (Peterson et al., 1975). As shown in Fig XXX, mutation frequencies of at least 10 times background have consistently achieved in both lines. Unexpectedly, both the spontaneous mutation frequency and the induced mutation frequency were somewhat higher in the p53+ line.

Molecular analysis of mutant clones A total of more than 200 thioguanine-resistant clones (putative *HPRT* mutants) have been collected, including spontaneous and induced mutants from both cell lines, and these are being characterized by Northern blot, RT-PCR and DNA sequencing. Twenty-three independent bleomycin-induced mutations from the p53+ line and twenty-five from the p53- line have thus far been identified. In both lines, a number of single-base-pair (-1) deletions were detected (Table YY and Fig. ZZ), nearly all of which were targeted to expected sites of bleomycin-induced double-strand cleavage ($p<0.003$ for all -1 deletions combined). The occurrence of such mutations, which is a hallmark of bleomycin-induced mutagenesis (Povirk et al., 1994), suggests that significant mutagenic DNA damage was induced in both cell lines by the drug treatment. As shown in Fig. ZZ, these -1 deletions probably arise by end-joining repair of blunt-ended (or, in a few cases, staggered) double-strand breaks, resulting in deletion of the base pair destroyed in the formation of the break. For both cell lines, there were also a number of mutants which showed deletion of one exon in the cDNA (most frequently exon 6), suggesting that in the active *HPRT* allele of these mutants, the 5′ or 3′ splice signal for that exon was either altered or deleted. Remarkably, skipping of exon 6 accounted for a third of both spontaneous and induced mutants derived from the p53- line, and because of their prevalence, further molecular analysis of these mutants is now planned. First, genomic DNA will be isolated and subjected to long-range PCR with primers upstream of exon 5 and downstream of exon 7, which should reveal any large (>1 kb) deletions (Van Houten et al., 1998). Mutants showing no such alterations will be subjected to PCR with more closely-spaced primers spanning exon 6, in order to detect any smaller deletions. Finally, for those mutants still showing no detectable alterations, portions of the exon 6 PCR products will be sequenced to detect any point mutations in the splicing signals.

Overall, the mutation spectra were qualitatively similar for p53+ and p53- cell lines. However, only the p53- cells yielded mutants for which no *HPRT* RT-PCR product could be generated (Table YY). These mutants may be reciprocal translocations or other large-scale rearrangements which abolish production of mature mRNA. Furthermore, it may be that these rearrangements occur only in p53- cells because such events are suppressed by normal p53 function. To determine the nature of these mutations and verify their occurrence only in p53- cells, RNA from all mutants is being subjected to Northern blot analysis, which will provide a quantitative measure of *HPRT* mRNA levels that should be less prone to possible artifacts than is RT-PCR. The chromosomes of any mutants found to be devoid of *HPRT* mRNA will be analyzed by SKY in order to screen for reciprocal translocations or other large-scale alterations of the X chromosome, as well as to determine whether any such mutants have acquired a global chromosomal instability (Morgan et al., 1998). In addition, DNA from these mutants will be analyzed by Southern blotting with an *HPRT* cDNA probe to directly assess large-scale deletions or rearrangements at the *HPRT* locus. Although Southern analysis is complicated to some extent by the present of a normal allele from the inactive X chromosome, digestion with a battery of restriction enzymes should reveal any alterations

in which some portion of the active *HPRT* locus is still retained. Mutants with no large-scale *HPRT* alterations as well as mutants with a complete deletion of the *HPRT* locus will both yield a normal *HPRT* banding pattern on Southern blots; however, these two possibilities can be distinguished by the use of methyl-sensitive restriction enzymes, which give altered restriction patterns for the active (unmethylated) and inactive (methylated) *HPRT* alleles (Yen et al., 1984).

In previous studies with Chinese hamster ovary (CHO-D422) cells, the spectra of mutations induced at the hemizygous *aprt* locus by both bleomycin (Povirk et al., 1994) and neocarzinostatin (Wang and Povirk, 1997) included about 10% highly conservative, interchromosomal reciprocal translocations, apparently resulting from misjoining of the exchanged ends of two double-strand breaks. Since the *HPRT* locus presents a target that is twenty times larger than *aprt*, the CHO result would predict that on the order of half of all *HPRT* mutations would be translocations. On the contrary, the data thus far suggest that none of the *HPRT* mutations in the p53+ line, and at most only a few in the p53- line, are translocations. Thus, translocations resulting from double-strand break misrepair appear to be suppressed by factors present in 184B5 cells but absent from CHO, and p53 may be one factor contributing to that suppression.

Cell Line	184B5 Spontaneous	184B5 Drug-induced	184B5-E6c6 Spontaneous	184B5-E6c6 Drug-induced
Base substitutions	3	11	0	2
Single-base-pair deletions	0	3	1	6
Exon skipping	2	4	4	8
Other deletions	0	3	3	2
Rearrangements	0	1	0	0
No RT-PCR Product	0	0	1	4
Total	5	22	9	22

Table I.. Classification of spontaneous and bleomycin-induced *HPRT* mutations in 184B5 cells and in the p53-deficient derivative 184B5-E6c6, based on sequence analysis of the cDNA.

KEY RESEARCH ACCOMPLISHMENTS

- Substantiation of the absence of apoptotic cell death in breast tumor cells exposed to radiation or adriamycin.

- Establishment of the concept that Vitamin D3 analogs can be utilized to promote apoptotic cell death in the breast tumor cells at least in part, through sensitization of the cell to apoptotic cell death.

- Development of a new model for enhancement of exogenous gene delivery and expression in breast tumor cells (utilizing estradiol or irradiation).

- First molecular analysis of mutagenesis by any agent in a human mammary cell line

- First demonstration of targeted small deletions by a radiomimetic drug (bleomycin) in a human cell line

- Preliminary evidence for the occurrence of bleomycin-induced large-scale gene rearrangements only in the p53- cell strain

REPORTABLE OUTCOMES

- Manuscripts

* Vrana, J.A., Kramer, L., Saunders, A.M., Zhang, X-F., Dent, P., Povirk, L.F., and Grant, S. Inhibition of PKC activator-mediated induction of $p21^{CIP1}$ and $p27^{KIP1}$ by deoxycytidine analogs in human myelomonocytic leukemia cells: relationship to apoptosis and differentiation. Biochemical Pharmacology 58: 121-131, 1999

* Wang, Z.,, Van Tuyle, G., Conrad, D., Fisher, P.B., Dent, P., and Grant, S. Dysregulation of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ increases the susceptibility of human leukemia cells (U937) to 1-β-D-arabinofuranosylcytosine-mediated mitochondrial dysfunction and apoptosis. Cancer Research 59: 1259-1267, 1999.

Wang, Z., Wang, S., Fisher, P.B., Dent, P., and Grant, S. Impact of dysregulation of the cyclin-dependent kinase inhibitor $p21^{CIP1}$ on leukemic cell differentiation induced by low concentrations of 1-β-D-arabinofuranosylcytosine. Submitted to Blood.

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* Jain PT, Seth P and Gewirtz, D.A. Estradiol enhances liposme-mediated uptake, preferential nuclear accumulation and functional expression of exogenous genes in MDA-MB231 breast tumor cells. Biochim Biophys Acta, In Press.

* Jain PT and Gewirtz, DA. Sustained improvement of liposomal gene delivery to human breast tumor cells by ionizing radiation. *Int J Radiat Biol 75: 217-223, 1999*

* Magnet, KJ and Gewirtz, DA. Influence of ionizing radiation on select proteins of the G1 checkpoint and apoptotic pathway: relationship to growth arrest and cell death in breast tumor cells with wild-type p53. Submitted to Int J Rad Biology.

* Sundaram, S and Gewirtz, DA. Enhancement of breast tumor cell sensitivity to ionizing radiation by Vitamin D3 and the Vit D3 analog, EB 1089. Radiation research. In Press.

Sundaram S and Gewirtz DA. Sensitization of breast tumor cells to adriamycin by the Vitamin D analog EB 1089. In Preparation.

* indicates that preprints or reprints have been provided with this annual report.

- Presentations

Chen, S. and Povirk, L.F.: Processing of terminally blocked DNA double-strand break ends *in vitro* and *in vivo*, and the role of DNA-PK. Invited presentation, 6th Intl. Workshop on Radiation Damage to DNA, Chapel Hill NC, April 17-21, 1999.

Yu, Y., and Povirk, L.F.: Genomic instability and gene rearrangements induced by radiomimetic antibiotic bleomycin in nontransformed 184B5 mammary epithelial cells. Presented at the annual meeting of the Environmental Mutagen Society, Mar. 27 - Apr 1, 1999, Washington D.C.

Magnet KJ and Gewirtz DA. Influence of ionizing radiation on proliferation and c-myc expression in twp p53 positive breast tumor cell lines. Presented at the Annual Meeting of the American Association for Cancer Research, March 28 - April 1, 1998, Philadelphia, PA.

Sundaram S and Gewirtz, D.A. EB 1089 enhances the antiproliferative effects of radiation in breast tumor cells. Presented at the Annual Meeting of the American Association for Cancer Research, March 28 - April 1, 1998, Philadelphia, PA.

- Funding applied for based on work supported by this award

We have recently submitted a clinical translational proposal to the Department of Defense Breast Cancer Research Program entitled: Utilization of Vitamin D analogs to enhance the response of breast cancer to chemotherapy (Adriamycin).

CONCLUSIONS

- One of our primary conclusions is that breast tumor cells are refractive to chemotherapy and radiotherapy induced apoptosis. The implication of this finding is that the recurrence of disease could be a consequence of the absence of apoptotic cell death in metastatic breast cancer. Consequently, we have modified our goals to develop approaches to enhance the sensitivity of the breast tumor cell to radiation and drugs such as adriamycin.

- We have developed two primary approaches which we ultimately hope to test both in an animal model system and, if successful, in the clinical setting. These approaches are to combine radiation or adriamycin with Vitamin D analogs which are not hypercalcemic. It is anticipated that these combinations could lead to more effective cell killing at conventional (or reduced) doses of drugs and radiation.

- We have also developed approaches for enhancing the uptake and expression of exogenous genes (using either high dose estradiol or radiation). These studies may ultimately have utility in the area of gene therapy to increase the delivery of genes which promote cell death. .

- We have shown that 184B5 is a suitable mammary epithelial cell line for molecular analysis of mutations, and that the combination of 184B5 and 184B5-E6c6 cell lines constitutes a suitable system for comparing mutagenesis in p53+ and p53-defective backgrounds. Most of the mutant collection has now been completed and the remainder of the project period will be devoted to mutant analysis. From the limited number of clones analyzed thus far, it is clear that targeted mutations are being induced by bleomycin in both lines, but it appears that large-scale rearrangements may occur only in the p53- line. Verification of this finding by Northern blot, combined with further molecular and chromosomal analysis of these mutant clones, may implicate p53 in suppressing particular types of errors in the repair of DNA double-strand breaks.

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Legends to Figures

Figure 1. Influence of adriamycin on proliferation of MDA-MB231 and T-47 D breast tumor cells. The two breast tumor cell lines were exposed to 1 micromolar adriamycin for 2 hours and viable cell number was monitored (based on trypan blue exclusion) at the indicated 24 hour intervals.

Figure 2. Suppression of c-myc expression in MCF-7 cells and lack of suppression in ZR-75-1 cells by ionizing radiation. P53 wild-type MCF-7 and ZR-75-1 breast tumor cells
were exposed to 10 Gy of ionizing radiation and Myc protein levels were monitored by Western blotting at the indicated times.

Figure 3. Relative sensitivity to adriamycin in MCF-7 and MDA-MB231 breast tumor cells. Growth inhibition by adriamycin after 72 hours was determined in 96 well microtitre plates by the MTT dye assay.

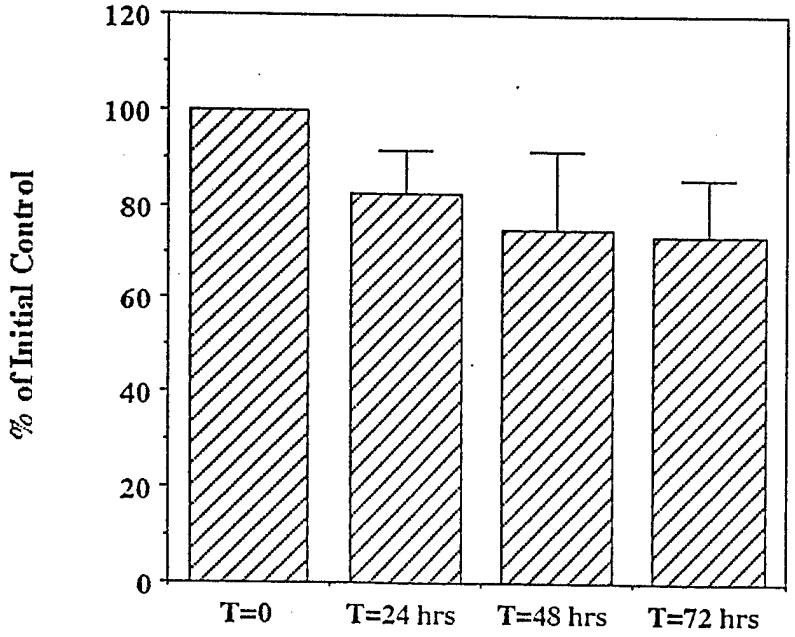
Figure 4. Development of MCF-7 breast tumor cell lines expressing antisense to p21.

Figure 5. Enhanced antiproliferative activity of the combination of EB 1089 or RO-23-7553 with adriamycin in MCF-7 breast tumor cells. Cells were treated with 100nM EB1089 or 200nM RO-23-7553 for 48 hours prior to adriamycin. Cell numbers were determined after an additional incubation time of 72 hours.

Figure 6 Survival and mutagenesis a the *HPRT* locus for 184B5 (p53+) or 184B5-E6c6 (p53-) cells treated with bleomycin in plateau phase. Each point is the mean ± SE for 3-6 independently treated cultures, from a total of 5 (184B5) or 2 (184B5-E6c6) experiments.

Figure 7. Bleomycin-induced targeted single-base-pair (-1) deletions. In (A), the bars show the locations of three independent -1 deletions from bleomycin-treated 184B5 or 184B5-E6c6 cells. Arrows show the expected sites of DNA double-strand cleavage, and different arrow styles serve to distinguish closely-spaced sites. Also shown are models for the generation of -1 deletions by end-joining repair of directly opposed (B) or staggered (C) double-strand breaks.

The Effect of 1 uM Adriamycin on T47D Breast Tumor Cells



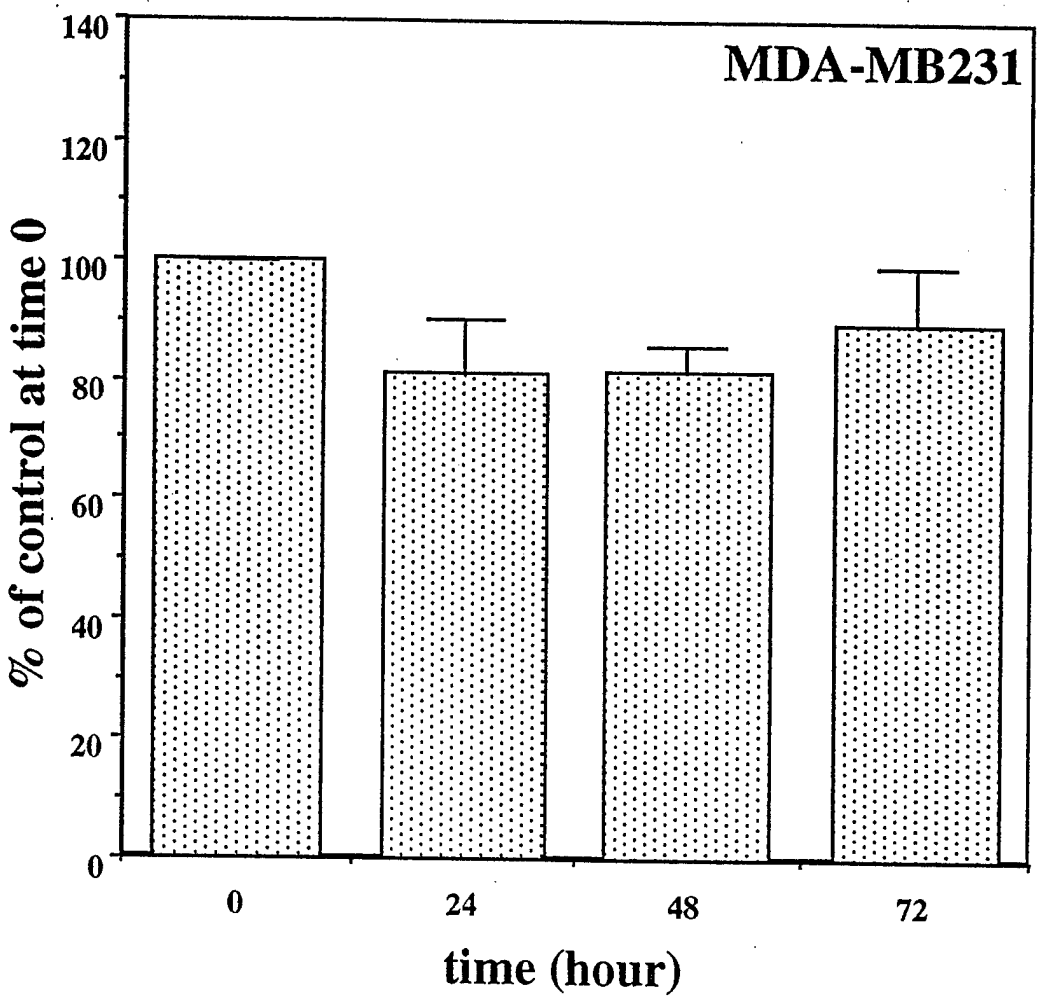


Figure 1. Influence of adriamycin on growth and viability of T-47D and MDA-MB231 breast tumor cells. Cells were exposed to 1μM adriamycin for 2-4 hours and viable cell number was monitored for 72 hours. Values are presented as percent of initial controls at time zero.

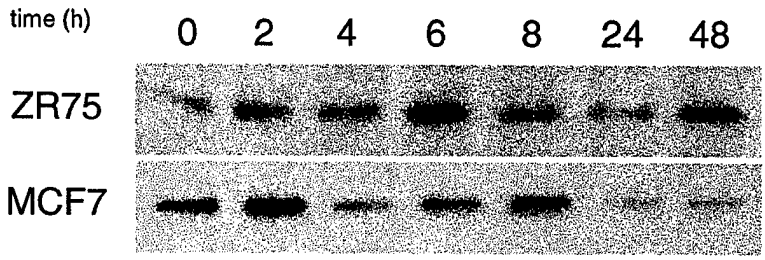


Figure 2. Suppression of Myc levels by ionizing radiation in MCF-7 breast tumor cells and lack of suppression in ZR-75-1 cells. Cells were exposed to 10 Gy of ionizing radiation and Myc levels assessed by Western blotting at the indicated times.

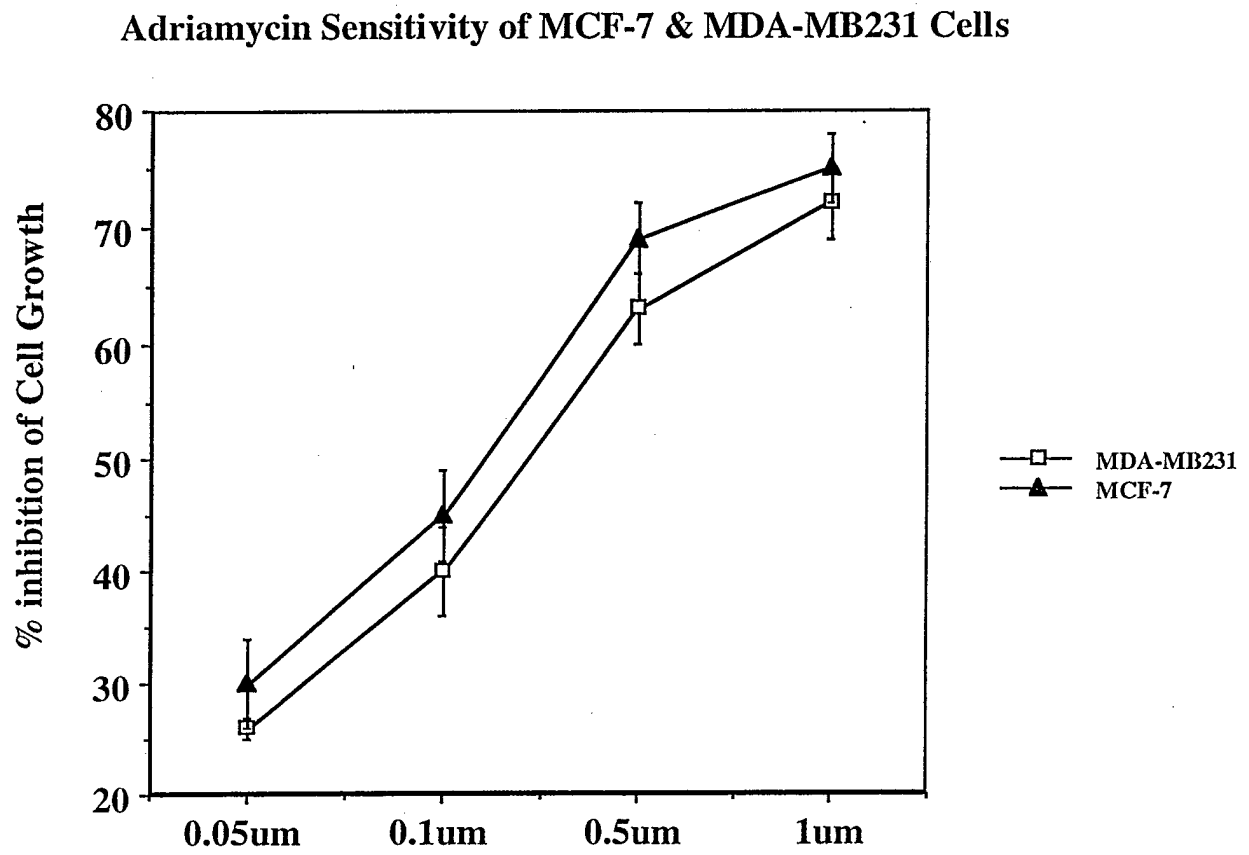
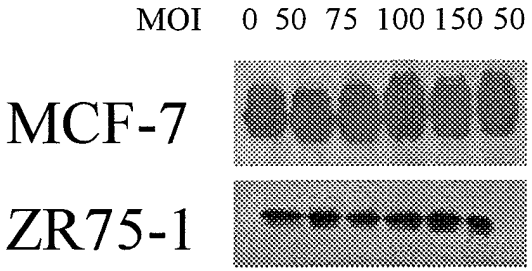
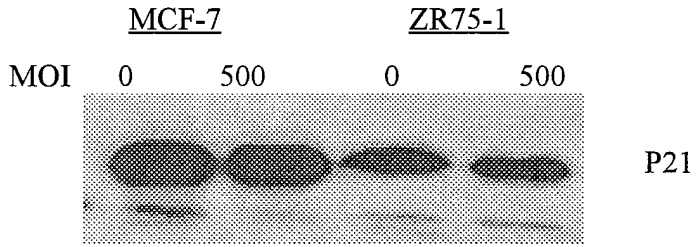


Fig 3 Comparative sensitivity to adriamycin in MCF-7 and MDA-MB231 cells. The dose response for antiproliferative activity was essentially identical in the two cell lines.

Infection of MCF-7 and ZR-75 cells with antisense waf1/cip1 adenoviral vectors



TEST 1



TEST 2

Transfection with an vector pREP4 containing the p21 WAF1/CIP1 coding region in the antisense orientation by electroporation

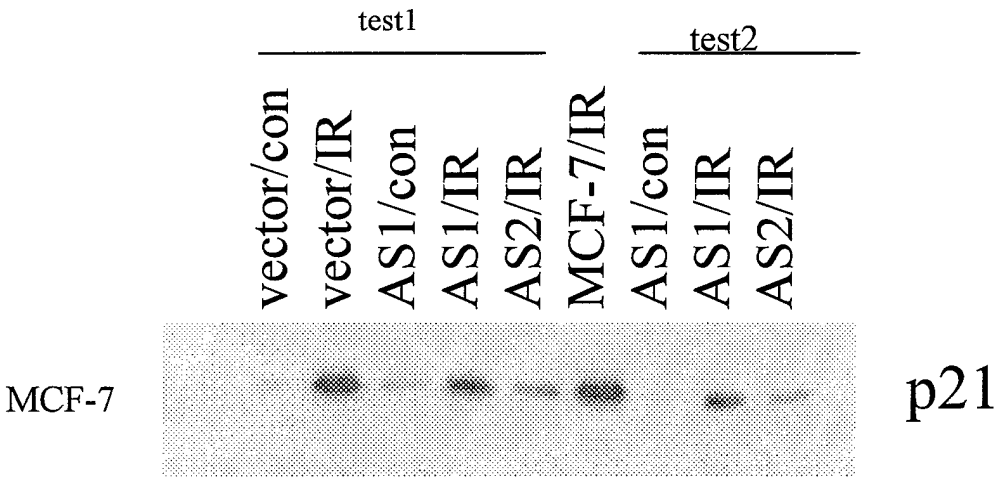
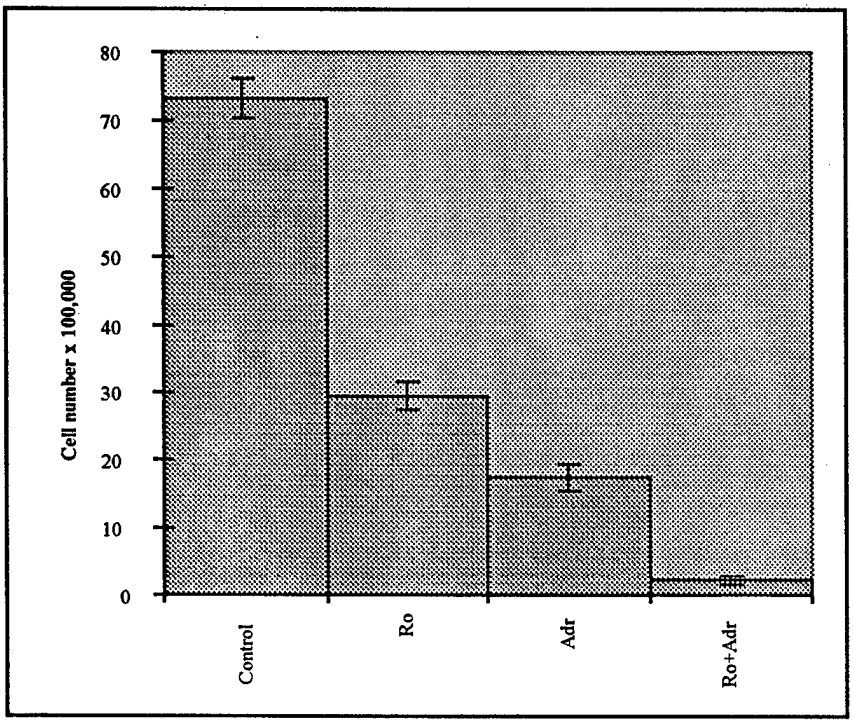


Figure 4

Cell number for Ro 23-7553, Adr and Ro+Adr treated MCF-7 cells



Cell number for EB 1089, Adr and EB 1089+Adr treated MCF-7 cells

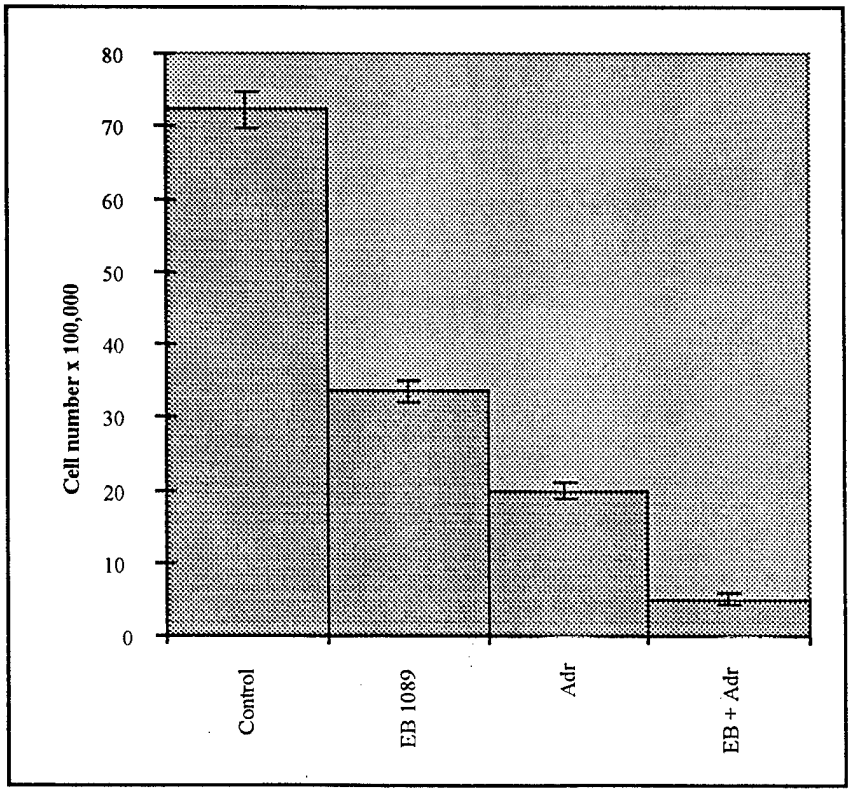


Figure 5

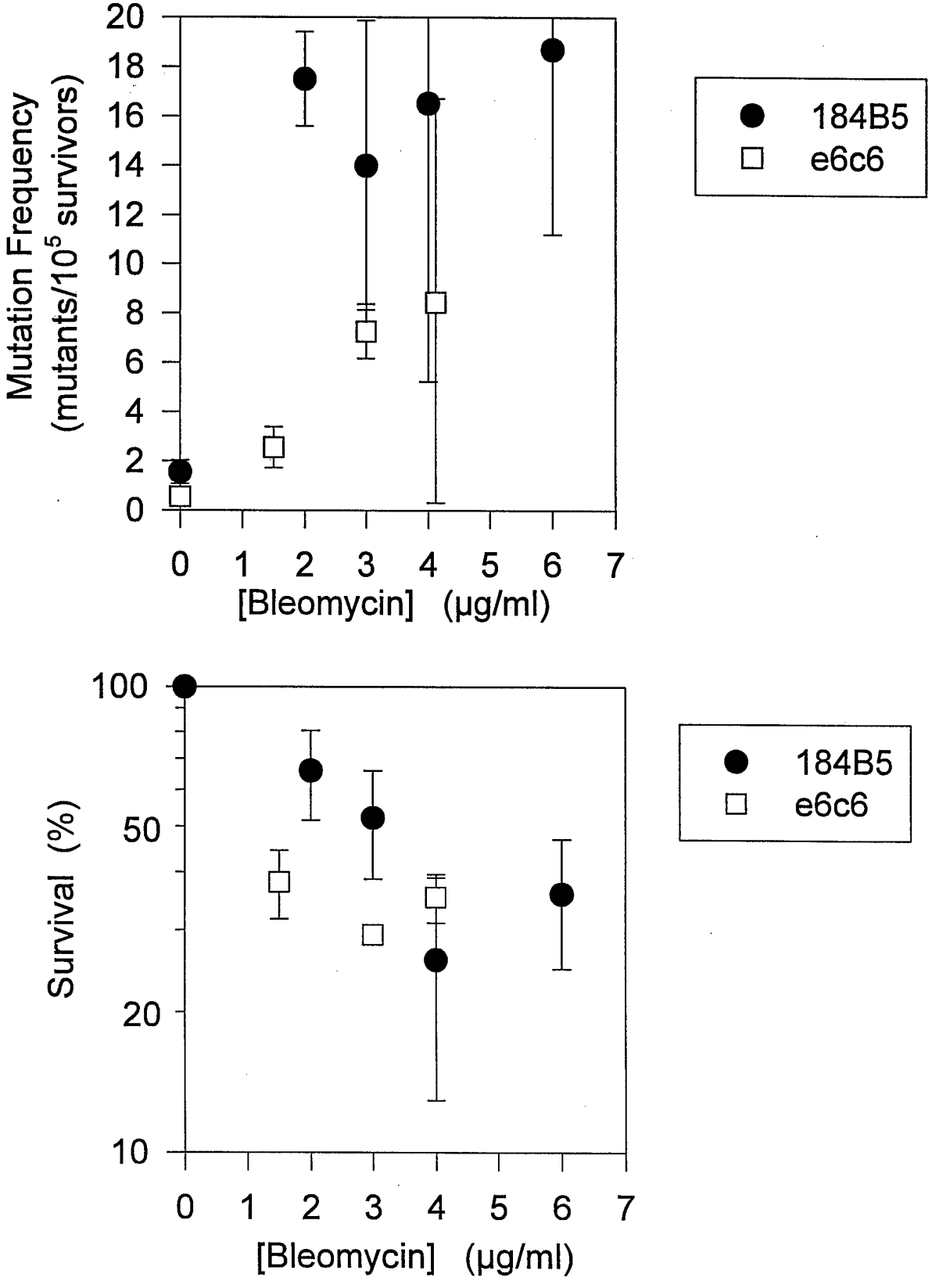

Mutation Frequency
(mutants/10^5 survivors)
20
18
16
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7
[Bleomycin] (µg/ml)
184B5
e6c6
Survival (%)
100
50
20
10
0 1 2 3 4 5 6 7
[Bleomycin] (µg/ml)
184B5
e6c6

Figure 6

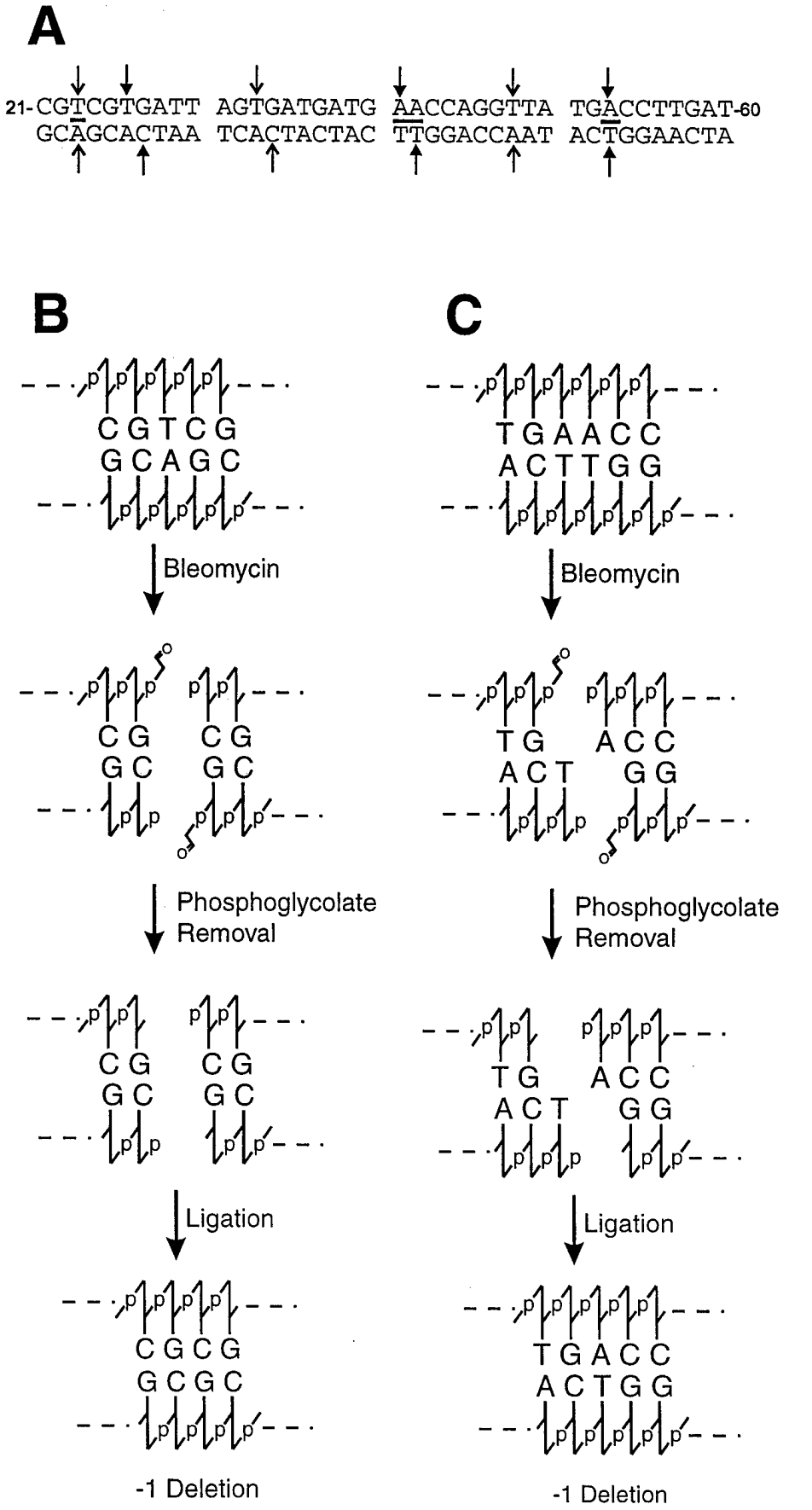

A
21-CGTCGTGATT AGTGATGATG AACCAGGTTA TGACCTTGAT-60
GCAGCACTAA TCACTACTAC TTGGACCAAT ACTGGAACTA
B
C G T C G
G C A G C
Bleomycin
C G C G
G C G C
Phosphoglycolate Removal
C G C G
G C G C
Ligation
C G C G
G C G C
-1 Deletion
C
T G A A C C
A C T T G G
Bleomycin
T G A C C
A C T G G
Phosphoglycolate Removal
T G A C C
A C T G G
Ligation
T G A C C
A C T G G
-1 Deletion

Figure 7

INT. J. RADIAT. BIOL 1998, VOL. 74, NO. 5, 561–564

Rapid communication

A highly conservative, cyclically permuted, non-homologous exchange among three unrelated DNA sequences in bleomycin-treated CHO cells

L. F. POVIRK

(Received 20 March 1998; accepted 5 June 1998)

Abstract. Molecular analysis of a bleomycin-induced rearrangement of the *aprt* gene in CHO cells revealed that it consisted of a nearly perfect three-way exchange among non-homologous sequences, consistent with a mechanism involving cyclically permuted misjoining of the six ends of three double-strand breaks.

Metaphase spreads of cells exposed to ionizing radiation (Lea 1946) or radiomimetic drugs (Vig and Lewis 1978) typically contain chromosome fragments as well as both reciprocal and non-reciprocal exchange-type aberrations. Based on this observation, it was proposed more than 50 years ago that the exchanges probably resulted from aberrant rejoining of the chromosome fragments (Lea 1946). In the simplest model, free DNA double-strand break ends are assumed to have a significant but limited diffusion radius in the nucleus, resulting in a finite possibility of being misjoined to the ends of other double-strand breaks in the DNA of different chromosomes in the same nucleus (Bender *et al.* 1974, Savage 1993). However, in the absence of molecular data, more complex models involving, for example, specific cooperative interactions among pairs of ends, extensive exonucleolytic single-strand exposure, aberrant homologous recombination among closely related sequences on different chromosomes, or other forms of homologous pairing, cannot be excluded (Savage 1989, Thacker *et al.* 1992, Lucas and Sachs 1993, Nicolàs *et al.* 1995).

For exchanges or other large-scale rearrangements that involve a known genetic locus, ligation-mediated PCR (figure 1) has greatly facilitated molecular analysis (Mizobuchi and Frohman 1993). Once a breakpoint in the known locus has been localized by PCR-based mapping, the breakpoint and a short segment of the unknown DNA segment beyond can be amplified and sequenced. This method was used recently for the analysis of several putative interchromosomal misjoining events, initially detected as mutations at the hemizygous *aprt* locus of bleomycin-treated plateau-phase CHO-D422 cells (Wang *et al.* 1997). Consistent with a mechanism involving the misjoining of exchanged double-strand break ends, most of these rearrangements were simple, highly conservative, non-homologous reciprocal exchanges, with breakpoints corresponding to potential sites of bleomycin-induced cleavage.

The single exception was mutant P5C (figure 2). With P5C DNA as a template, PCR, with a primer from each of the non-*aprt* sequences beyond the

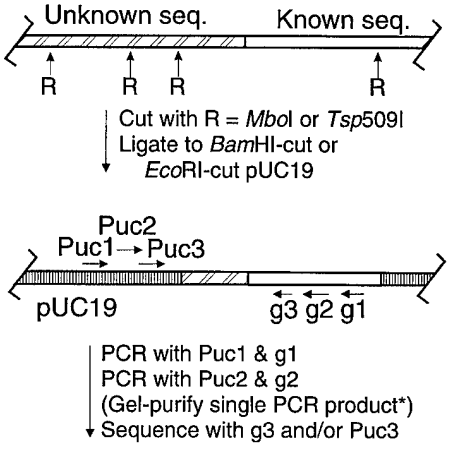


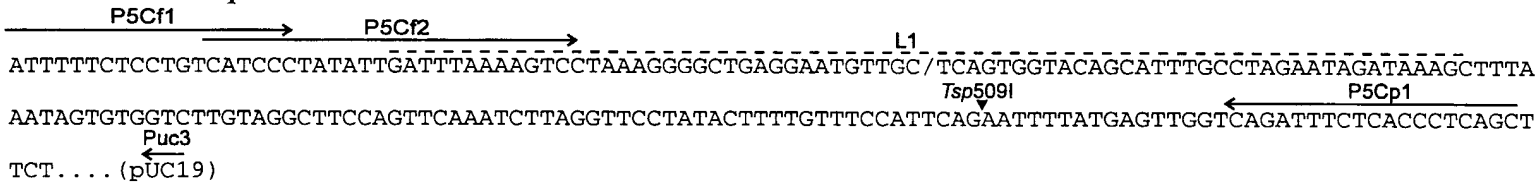
Figure 1. Genomic walking by ligation-mediated PCR. Genomic DNA is cleaved and ligated to an anchor (pUC19) having a matching cohesive end. Nested amplification, with pUC19 (Puc) primers and genomic (g) primers from the region of known sequence, allows recovery and sequencing of an adjacent unknown DNA segment (e.g. the sequences beyond a rearrangement junction). The restriction sites (R) should occur every ~256 bp on average and need not be known beforehand. *Gel fractionation is usually not required if a genomic primer (g3) is used for sequencing.

Department of Pharmacology and Toxicology, Medical College of Virginia, Virginia Commonwealth University, PO Box 980230, MCV Station, Richmond, VA 23298, USA. e-mail: LPOVIRK@gems.vcu.edu

A. Downstream *aprt* junction:

B. Upstream *aprt* junction:

C. Parental sequence 1:



D. Parental sequence 2:

E. Third junction:

Figure 2. Sequence analysis of the P5C rearrangement. Using nested ligation-mediated PCR (Mizobuchi and Frohman 1993, Zhou *et al.* 1997), parental sequences (C and D) involved in the rearrangement with *aprt* were obtained by extending sequences previously shown to be linked to *aprt* in the mutant (A and B). The third junction (E) was then amplified from mutant DNA and sequenced (see text for details). Bold lettering indicates *aprt* sequences, and lightface Roman and italic lettering indicate the two non-*aprt* parental sequences. Slashes indicate breakpoints in the parental sequences or newly formed junctions in the mutant sequences, and the lower-case *c* in (D) indicates a base that was lost in the exchange. Leftward facing primers indicate the complement of the sequence shown. Dashed lines indicate rodent short B1 and long L1 interspersed repeat elements, which were the only homologies detected in a search of Genbank.

breakpoints, directed towards the breakpoint (e.g. primers P5Cf1 and P5Cr6), failed to generate any product, suggesting that the two non-*aprt* sequences were not contiguous in the parent cell line and that the rearrangement was not a simple reciprocal exchange. The lack of PCR product was apparently not due to a defect in the primers, since each could regenerate a junction fragment of the expected length using mutant DNA as a template and a second primer from *aprt*.

To recover the segment of non-*aprt* parental sequence 1 that had been replaced by *aprt* sequences (figure 2A), non-mutant DNA was cleaved with *Mbo*I (↓GATC) or *Tsp*509I (↓AATT) and ligated to *Bam*HI-cut or *Eco*RI-cut dephosphorylated pUC19. Nested amplification was performed with primers Puc1 and P5Cf1, and then with primers Puc2 and P5Cf2 (where Puc primers correspond to sequences within the pUC19 anchor; Wang *et al.* 1997), yielding predominant PCR products of ~220 bp (*Mbo*I) and ~190 bp (*Tsp*509I). Each of these fragments was isolated from a low-melting-point agarose gel and sequenced with a third nested primer from the pUC19 anchor (Puc3: TGTAAAACGACGGCCAGTG). In each case, the portion of the sequence farthest from the Puc3 sequencing primer was identical to the sequence beyond the downstream *aprt* breakpoint in mutant P5C, and the new sequences nearer the primer were, except for the start site, the same for the 220 and 190 bp fragments. The stagger in start sites was consistent with the position of a *Tsp*509I site in the sequence derived from the *Mbo*I-cut DNA (figure 2C). Thus, it is inferred that this sequence corresponds with one of the original parental sequences involved in the *aprt* rearrangement.

Similarly, non-*aprt* parental sequence 2 was obtained by subjecting the same ligated DNA to nested amplification with primers Puc1 and P5Cr6, and then with primers Puc2 and P5Cr5. Again, sequencing of gel-purified ~290 bp (*Mbo*I) and ~250 bp (*Tsp*509I) PCR products, using the Puc3 primer, gave identical sequences but with different start sites (figure 2D). However, the sequence at the breakpoint was unreadable due to apparent heterogeneity of a $(CA)_n$ microsatellite located between the primer and the breakpoint. Nevertheless, enough of parental sequence 2 was obtained to design primer P5Cp2, which was used along with primer P5Cr6 to amplify the breakpoint region of parental sequence 2 directly from non-mutant genomic DNA in one step. This reaction yielded the expected predominant ~130 bp product, which was gel-purified. Sequencing of this product with primer P5Cr6 yielded an unambiguous breakpoint sequence, while sequencing with primer P5Cp2 gave a pattern consistent with two identical junction sequences staggered by 2 bp, thus confirming the heterogeneity of the microsatellite.

As shown in figure 2, if the rearrangement in P5C were a precise, cyclically permuted exchange of the three non-homologous sequences (*aprt* and parental sequences 1 and 2), then amplification of mutant DNA with primers P5Cp2 and P5Cp1 should yield a ~205 bp fragment containing the third rearrangement junction, linking the two non-*aprt* sequences. As shown in figure 3, such a fragment could indeed be generated from DNA of mutant P5C, but not from DNA of the parental cell line. Following elution from the gel, this fragment was sequenced with each of the two PCR primers, and the results confirmed that it contained the predicted third junction of the rearrangement (figure 2E). Thus, as summarized in figure 4, the rearrangement was in fact a cyclically permuted exchange among the three unrelated DNA sequences. Like the reciprocal exchanges detected previously (Wang *et al.* 1997), this three-way exchange was highly conservative, with not more than a single base pair being lost from any of the parental sequences. Other than a high incidence of TC dinucleotides and $(TC)_n$ repeats near the breakpoints, there were no significant homologies between the parental sequences, and no evidence of interactions between single-stranded regions. However, there was a single one-base-pair non-templated addition, a feature not seen in any of the six bleomycin-induced reciprocal exchanges. Also, whereas all twelve breakpoints of the six reciprocal exchanges corresponded with expected sites of bleomycin-induced double-strand cleavage, one of the breakpoints in the three-way

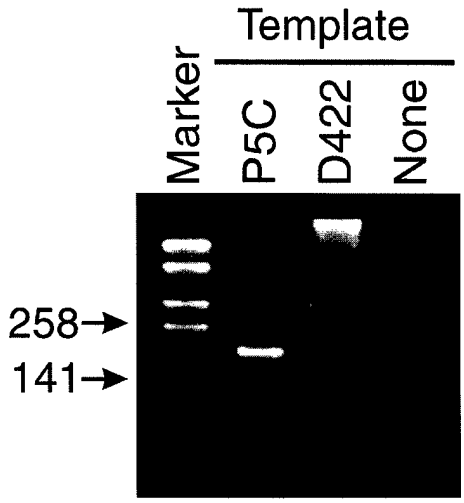


Figure 3. Amplification of a fragment, containing the third junction, from DNA of mutant P5C, but not from DNA of the D422 parental strain.

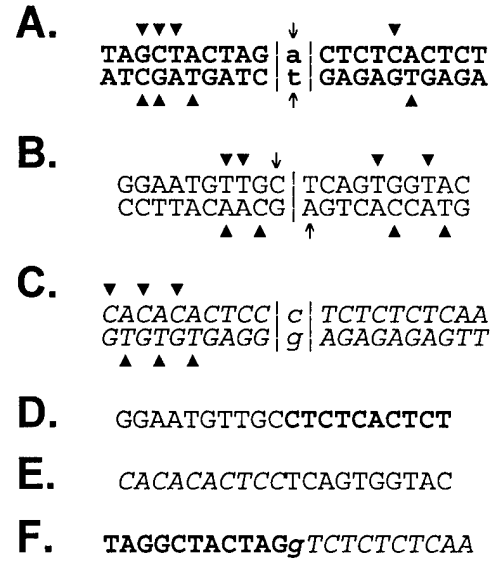


Figure 4. Summary of breakpoints (|) in *aprt* (A) and in the other parental sequences (B–C), and newly formed junctions (top strand only) in the rearrangement (D–F). Arrows indicate expected sites of bleomycin-induced double-strand cleavage that would be consistent with the observed breakpoints, and triangles indicate sites that would not. As described by Povirk *et al.* (1989), potential double-strand cleavage sites consist of cleavage in one strand at any G–*Py* sequence, plus cleavage in the complementary strand either directly opposite or on a one-base 5′ stagger, depending on the sequence; breaks in both strands bear 5′-phosphate and 3′-phosphoglycolate termini. Lower-case letters in (A) and (C) indicate base pairs, which were unambiguously lost in the rearrangement. Lower-case *g* in (F) indicates an apparent non-templated insertion. Note that the *C* just left of the junction in (E) could have originated from either parental sequence.

exchange did not (figure 4). Thus, if the exchange was the result of misjoining of three double-strand breaks, one of the breaks must have arisen by some mechanism other than the direct action of bleomycin.

It was shown previously by fluorescence *in situ* hybridization (FISH) that sequences immediately upstream and downstream of *aprt* were separated to different chromosomes in the P5C mutant (Wang *et al.* 1997). This result implies that, in the parental CHO-D422 cells, either parental sequence 1 or parental sequence 2 (or both) was initially on a different chromosome than *aprt*, and that the exchange produced at least one chromosomal translocation. In irradiated cells, cyclically permuted three-way exchanges were detected previously at the chromosomal level using three-colour FISH (Lucas and Sachs 1993), and these events were taken as evidence that exchanges in general probably result from independent binary interactions between two DNA ends, rather than any concerted interactions among pairs of ends.

The present results, which to the author's knowledge describe the first three-way exchange of any kind to be fully sequenced, support this proposal in that they show that at the DNA sequence level the three-way exchange was qualitatively similar to the simple reciprocal exchanges generated under the same conditions. In particular, neither event appeared to involve any homology-dependent processing.

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Dysregulation of the Cyclin-dependent Kinase Inhibitor p21$^{WAF1/CIP1/MDA6}$ Increases the Susceptibility of Human Leukemia Cells (U937) to 1-β-D-Arabinofuranosylcytosine-mediated Mitochondrial Dysfunction and Apoptosis[1]

Zhiliang Wang, Glenn Van Tuyle, Daniel Conrad, Paul B. Fisher, Paul Dent, and Steven Grant[2]

Departments of Medicine [Z. W., S. G.], Pharmacology [S. G.], Microbiology [D. C., S. G.], Radiation Oncology [P. D.], and Biochemistry [G. V., S. G.], Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0230, and the Departments of Pathology and Urology, Columbia University College of Physicians and Surgeons, New York, New York 10032 [P. B. F.]

ABSTRACT

The effects of dysregulation of the cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ on the apoptotic response of U937 monocytic leukemia cells to 1-β-D-arabinofuranosylcytosine (ara-C) were examined. After a 6-h exposure to 1 μM ara-C, cells stably transfected with a p21$^{WAF1/CIP1}$ antisense construct were significantly more sensitive to the induction of classic apoptotic morphology, DNA fragmentation, caspase-3 activation, poly(ADP-ribose) polymerase degradation, and underphosphorylation of the retinoblastoma protein (pRb) than their empty-vector counterparts. Enhanced susceptibility of antisense-expressing cells to ara-C was accompanied by a corresponding reduction in clonogenic and suspension culture growth. The increased sensitivity of these cells to ara-C-mediated lethality could not be attributed to cytokinetic perturbations, nor did ara-CTP formation or (ara-C)DNA incorporation differ significantly between the cell lines. Moreover, synchronization of p21 antisense-expressing cells in S-phase by aphidicolin block resulted in a further increase in ara-C-mediated apoptosis, suggesting enhanced drug sensitivity of the S-phase cell fraction. After exposure to ara-C, p21 antisense-expressing cells displayed a greater decline in mitochondrial membrane potential ($\Delta\psi_m$) and generation of reactive oxygen species than their empty-vector counterparts, as well as early potentiation (*e.g.*, within 2–4 h) of cytochrome *c* release into the cytosolic S-100 fraction. Lastly, ara-C-mediated increases in mitogen-activated protein kinase activity over basal levels were attenuated in p21 antisense-expressing cells. Collectively, these findings indicate that dysregulation of the cyclin-dependent kinase inhibitor p21$^{WAF1/CIP1}$ increases the susceptibility of U937 human leukemia cells to ara-C-related lethality, and this phenomenon occurs as a relatively early event that is independent of cell cycle or pharmacodynamic factors and is associated with mitochondrial perturbations implicated in activation of the apoptotic protease cascade.

INTRODUCTION

The CDKI[3] p21$^{WAF1/CIP1/MDA6}$ belongs to a group of cell cycle regulatory proteins that includes p27^{KIP1} and p57^{KIP2} (1, 2). These proteins inhibit the activity of cyclin/CDK complexes and consequently play important roles in cell cycle arrest events accompanying DNA damage and cellular maturation (3). p21$^{WAF1/CIP1}$ has been referred to as a universal CDKI in view of its ability to inhibit multiple CDKs, including CDK1 (p34^{cdc2}), CDK2, and CDK4/6 (4). After DNA damage, the p53-dependent induction of p21$^{WAF1/CIP1}$ leads to G_1 arrest, permitting cells to undergo DNA repair, or if the damage is extensive, apoptosis (5). However, p21$^{WAF1/CIP1}$ is also induced in p53-null human leukemia cells exposed to differentiation-inducing agents such as tumor-promoting phorboids (*e.g.*, PMA; 6, 7).

In addition to its role in checkpoint regulation, there is accumulating evidence that p21$^{WAF1/CIP1}$ and other CDKIs may have a significant impact on the response of neoplastic cells to cytotoxic agents. For example, p21$^{WAF1/CIP1}$-deficient colorectal carcinoma cells have been shown to be more sensitive to a variety of DNA-damaging agents and ionizing radiation than their wild-type counterparts, a phenomenon attributed to uncoupling of S-phase and mitosis (8, 9). More recently, inducible expression of p21$^{WAF1/CIP1}$ has been found to reduce the sensitivity of glioblastoma cells to nitrosoureas and cisplatin (10). These findings are compatible with the results of studies demonstrating that other CDKIs, including p27 and p16, decrease the susceptibility of tumor cells to hydroperoxycyclophosphamide- and dexamethasone-mediated apoptosis, respectively (11, 12). Aside from the possibilities that CDKI dysregulation promotes inappropriate cell cycle traverse after cytotoxic drug insult (7) or interferes with DNA repair (13), the mechanism(s) by which CDKIs modulate drug sensitivity remains to be fully elucidated.

The antimetabolite ara-C, a highly active agent used in the treatment of acute leukemia, effectively induces apoptosis in leukemic cells (14). It does not, however, induce p21, at least in cells lacking functional p53 (6). Previously, we described the effects of stable transfection of human HL-60 promyelocytic leukemic cells with a p21$^{WAF1/CIP1}$ antisense construct on responses to the tumor-promoting PMA (15) and have recently extended these findings to the human myelomonocytic leukemic cell line U937 (16). Dysregulation of p21$^{WAF1/CIP1}$ in these cells has a variety of downstream consequences, including impairment in the ability of PMA to inhibit the activity of CDK2, dephosphorylate the retinoblastoma protein (pRb), induce G_1 arrest, and trigger a cellular maturation program (16). Disruption of p21$^{WAF1/CIP1}$ function did not, however, appreciably sensitize HL-60 cells to ara-C-related antiproliferative effects (15). The purpose of the present studies was to characterize the effects of p21$^{WAF1/CIP1}$ dysregulation on the apoptotic response of myelomonocytic leukemia cells to ara-C to identify the factor or factors that might be responsible for alterations in drug sensitivity. Our results indicate that in marked contrast to HL-60 cells, interference with p21$^{WAF1/CIP1}$ induction dramatically increases the susceptibility of U937 cells to ara-C-mediated apoptosis, and that this effect that occurs very early in the course of drug exposure. Furthermore, our results suggest that this phenomenon involves lowering of the threshold for ara-C-induced mitochondrial dysfunction and cytochrome *c* release, events postu-

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[2] To whom requests for reprints should be addressed, at Medical College of Virginia, MCV Station Box 230, Richmond, VA 23298. Phone: (804) 828-5211; Fax: (804) 828-8079.

[3] The abbreviations used are: CDKI, cyclin-dependent kinase inhibitor; PMA, phorbol-12 myristate-13 acetate; ara-C, 1-β-D-arabinofuranosylcytosine; ara-CTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; APH, aphidicolin; PBS-T, PBS-Tween; CDK, cyclin-dependent kinase; PARP, poly(ADP-ribose) polymerase; $DiOC_6$, 3,3′-dihexyloxacarbocyanin iodide; DHR 123, dihydrorhodamine 123; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase.

lated to represent critical initiators of the apoptotic protease cascade (17, 18).

MATERIALS AND METHODS

Cells. The myelomonocytic leukemia cell line U937 was derived from a patient with histiocytic lymphoma (19) and was obtained from American Type Culture Collection. Cells were cultured in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) containing 10% FCS and supplemented with penicillin and streptomycin, sodium pyruvate, MEM essential vitamins, and glutamate as described previously (16). Cells were maintained in a 37°C, 5% CO_2, fully humidified incubator and passed twice weekly.

To obtain antisense-expressing lines, cells were transfected by electroporation with either an empty pREP4 vector (Invitrogen, Carlsbad, CA) or a pREP4 vector containing the $p21^{WAF1/CIP1}$ coding region in the antisense orientation, as described previously (15). After selection in hygromycin (Life Technologies, Inc.; 400 μg/ml), individual cells were cloned after limiting dilution. Several clones were then selected that exhibited the greatest attenuation of $p21^{WAF1/CIP1}$ expression when exposed to 10 nM PMA for 24 h. Two such clones, designated U937/p21AS(F4) and U937/p21AS(B8), as well as a clone containing the pREP4 empty vector (U937/pREP4), were used in all subsequent experiments. All experiments were performed using logarithmically growing cells (cell density 3–5 $\times$ 10^5 cells/ml). Cells were tested for *Mycoplasma* contamination using the Gen-Probe kit (Gen-Probe, La Jolla, CA) and found to be negative.

Drugs and Chemicals. ara-C was purchased from Sigma Chemical Co. (St. Louis, MO) and maintained as a dry power at −20 C. It was reformulated in PBS before use. ara-CTP was purchased from Sigma, stored desiccated at −20°C, and formulated in water before use. PMA and APH were also purchased from Sigma, diluted in DMSO, and stored frozen under light-protected conditions at −20°C before use. In no case did the final DMSO concentration exceed 0.1%, nor did vehicle controls alter apoptotic or differentiation responses. 5-[^{3}H]ara-C (24 Ci/mmol) was purchased from Amersham Radiochemicals (Arlington, IL). $DiOC_6$, carbamoyl cyanide *m*-chlorophenylhydrazone, and DHR 123 were purchased from Molecular Probes (Eugene, OR).

Assessment of Apoptosis. Apoptotic cells were evaluated by morphological criteria as reported previously (20). After treatment of cells, cytospin preparations of the cell suspensions were fixed and stained with Wright-Giemsa. Cell morphology was evaluated by light microscopy, and apoptotic cells were identified by the the appearance of cell shrinkage, nuclear condensation, and/or the appearance of membrane-bound apoptotic bodies. Five to seven randomly selected fields were evaluated for each condition, encompassing a total of at least 500 cells. Alternatively, the appearance of oligonucleosomal DNA fragmentation characteristic of apoptosis was determined by agarose gel electrophoresis as described earlier (20). After drug exposure, pelleted cells (2 $\times$ 10^7 cells/condition) were lysed in 0.1% NP40, 10 mM Tris-HCl, and 25 mM EDTA (pH 7.4) containing 200 μg/ml Proteinase K and incubated for 24 h at 56°C. The lysates were then centrifuged at 48,000 $\times$ *g* for 45 min, and the supernatant was adjusted to 200 μg/ml RNase A for 4 h at 37°C. DNA unassociated with intact chromatin residing in the supernatant was then resolved by agarose gel electrophoresis at 6 V/cm in 1$\times$ TBE buffer (Tris-borate/EDTA) for 3 h on 1.8% agarose gels impregnated with ethidium bromide. Each lane was loaded with a volume of cell lysate corresponding to 2 $\times$ 10^6 cells, rather than with a fixed quantity of DNA. To permit estimation of fragment size, 100-bp DNA reference preparations were run in parallel.

Cell Viability. Logarthmically growing U937/pREP4 and p21AS cells were exposed to ara-C for 6 h, washed thoroughly in drug-free medium, and incubated for an additional 24 h in fresh medium. At the end of this time, the cells were enumerated by hemocytometer, and the number of viable cells was determined based upon their exclusion of 0.4% trypan blue dye.

Clonogenic Assay. After drug treatment, cells were enumerated using a hemacytometer, washed free of drug (three times) in 1$\times$ fetal bovine serum-free media, and plated in triplicate in 12-well plates (Costar) containing 500 cells/well, 1 ml of media, 20% fetal bovine serum, and 0.3% Bacto agar (Difco, Detroit, MI; 20). Plates were incubated in a fully humidified atmosphere of 95% air and 5% CO_2 at 37°C, and colonies, consisting of groups of $\geq$50 cells, were scored 10–12 days after plating.

Western Blot Analysis. Equal amounts of protein (25 μg) were separated by SDS-PAGE [5% stacker and 6% (PARP and pRb) or 12% (p21, bcl-2, bcl-xl, bax, and CPP32) or 15% (cytochrome *c*)] and electroblotted onto nitrocellulose as described previously (15). The blots were then blocked in PBS-T (0.05%) and 5% milk for 1 h with the appropriate dilution of primary antibody: PARP (1:10000; Biomol Research Laboratories, Plymouth Meeting, PA); pRb (1:2000; PharMingen, San Diego, CA); bcl-2 (1:2000; Dako, Copenhagen, Denmark); bcl-x_L (1:1000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA); bax (1:2000; Santa Cruz Biotechnology); p21cip1 (1:500; Transduction Laboratories, Lexington, KY); CPP32 (1:2000; Transduction Laboratories); cytochrome *c* (1:2000; PharMingen); c-Myc (provided by Dr. John Cleveland, St. Jude Children's Research Hospital, Memphis, TN; 1:2000); and actin (1:2000; Sigma). A murine IgG1 antibody (G99–549) that primarily reacts with underphosphorylated pRb species was obtained from PharMingen and used at a concentration of 1:2000. Blots were washed two times for 10 min in PBS-T and then incubated with a 1:2000 dilution of peroxidase-conjugated secondary antibodies (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) in PBS-T for 1 h at 22°C. Blots were again washed two times for 10 min in PBS-T and then developed by enhanced chemiluminescence (Pierce).

Preparation of S-100 Fractions and Assessment of Cytochrome *c* Release. U937 cells expressing antisense $p21^{WAF1/CIP1}$ and empty vector-containing controls were harvested after drug treatment by centrifugation at 600 $\times$ *g* for 10 min at 4°C. The cytosolic S-100 fraction was prepared as described (18), with minor modifications. Cell pellets were washed once with ice-cold PBS and resuspended in five volumes of buffer A [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, and 250 mM sucrose]. After being chilled for 30 min on ice, the cells were disrupted by 15 strokes of a glass homogenizer. The homogenate was centrifuged twice to remove unbroken cells and nuclei (750 $\times$ *g*, 10 min, 4°C). S-100 fractions (supernatants) were then obtained by centrifugation at 100,000 $\times$ *g*, 60 min at 4°C. All steps were performed on ice or 4°C. Cytochrome *c* release into the S-100 fraction for each condition was assessed by Western blot analysis of the resulting fractions as detailed above.

Cell Cycle Analysis. After drug treatment, cells were pelleted at 500 $\times$ *g* and resuspended in 70% ethanol. The cells were incubated on ice for at least 1 h and resuspended in 1 ml of cell cycle buffer (0.38 mM sodium citrate, 0.5 mg/ml RNase A, and 0.01 mg/ml propidium iodide) at a concentration of 10 $\times$ 10^5 cells/ml. Samples were stored in the dark at 4°C until analysis (usually within 24 h), using a Becton Dickinson FACScan flow cytometer and ModFit LT 2.0 software (Verity Software, Topsham, ME).

Cell Cycle Synchronization. Synchronization of cells within G_1-S phase of the cell cycle was achieved using an APH block. Cells were treated with 0.15 μg/ml for 24 h and washed in drug-free medium three times before resuspension. Synchronization was confirmed by propidium iodide staining and flow cytometry (see above). Cell viability immediately after release from APH block was routinely >90%, and the percentage of cells in G_1-S at this interval was consistently 85–90%. Moreover, this concentration of APH was found to be without discernible biological effect in U937 cells. Synchronized cells were used for experimentation immediately to prevent cells from reentering the cell cycle.

ara-CTP Formation. Logarithmically growing $p21^{WAF1/CIP1}$ antisense-expressing cells and cells containing the empty vector were exposed to 1 μM ara-C for 6 h, centrifuged for 10 min at 400 $\times$ *g* at 4°C, and washed twice with PBS, and the pellet precipitated with trichloroacetic acid as reported previously (21). Neutralized acid-soluble material was extracted using freon-octylamine, treated with sodium periodate to eliminate ribonucleotides, and subjected to high-pressure liquid chromatographic analysis using a Bio-Rad Model 700 Workstation, a Beckman Medel 260 detector, a Waters Partisil SAX column, and a K_2HPO_4 gradient elution system. Peaks coeluting with authentic ara-CTP standards were integrated automatically and expressed as pmol ara-CTP/10^6 cells.

ara-C(DNA) Incorporation. Assessment of incorporation of ara-C residues into the DNA of U937/p21 antisense-expressing and empty vector control cells was performed using a method described previously (21). After incubation of cells with 1 μM 5-[^{3}H]ara-C for 6 h, cells were pelleted and lysed, and DNA was isolated by phenol-chloroform extraction and ethanol precipitation. DNA was then resuspended in TE buffer, quantitated spectrofluorometrically, and aliquots were added to an aqueous scintillation cocktail before scintillation counting. Values are expressed as pmol 5-[^{3}H]ara-C incorporated/ng DNA.

Assessment of Mitochrondrial Membrane Potential ($\Delta\Psi_m$) and ROS. Mitochrondrial membrane potential was monitored using $DiOC_6$ (17). In addition, DHR 123 was used to measure ROS (22). In the presence of ROS, DHR 123 is oxidized to the highly fluorescent rhodamine 123. For each condition, 4×10^5 cells were incubated for 15 min at 37°C in 1 ml of 40 nM $DiOC_6$ (3) or 1 μM of DHR123 and subsequently analyzed using a Becton Dickinson FACScan cytofluorometer with excitation and emission settings of 488 and 525 nm, respectively. Control experiments documenting the loss of $\Delta\Psi_m$ and generation of ROS were performed by exposing cells to 5 μM carbamoyl cyanide *m*-chlorophenylhydrazone (15 min, 37°C), an uncoupling agent that abolishes the mitochondrial membrane potential, or 10 nM H_2O_2, respectively. Control gates were set as shown in the figures, and loss of $\Delta\psi_m$ or generation of ROS was quantified using the Cyclops program (Cytomation, Boulder, CO).

Determination of SAPK (JNK) and MAPK Activities. A method described previously was used (23). Pelleted cells were washed in PBS, repelleted, and flash-frozen. Cell pellets were lysed in the 25 mM sodium β-glycerophosphate (pH 7.4), containing 5 mM EGTA, 5 mM EDTA, protease inhibitors (5 mM benzamidine, 1 mM phenylmethysulfonyl fluoride, 1 mg/ml soybean trypsin inhibitor, 40 μg/ml pepstatin, 40 μg/ml chymotrypsinogen, 40 μg/ml E64, 40 μg/ml aprotinin, and 1 μM microcystin LR), phosphatase inhibitors (1.0 mM trisodium orthovanadate and 1.0 mM tetrasodium PP_i), 0.05% (w/v) sodium deoxycholate, 1% (v/v) Triton X-100, and 0.1% (v/v) 2-mercaptoethanol. Lysates were clarified by centrifugation at 5000 $\times$ *g* at 4°C for 5 min. SAPK/MAPK was immunoprecipitated from clarified lysates with protein A/agarose-conjugated antibody/antisera. JNK activity was then assayed after immunoprecipitation of p54-JNK1/p46-JNK2 using glutathione-*S*-transferase/c-Jun 1–169 as substrate. Alternatively, MAPK activity was assayed after immunoprecipitation of p42-ERK1/p44-ERK2 using myelin basic protein as substrate. Preimmune controls were also run to ensure selectivity of substate phosphorylation. Reaction mixtures consisted of immunoprecipitated enzyme, substrate, and 0.1 mM [γ-^{32}P]ATP(5000 Ci/pmol) in 25 mM sodium β-glycerophosphate (pH 7.4), containing 15 mM $MgCl_2$, 100 μM trisodium orthovanadate, 0.01% (v/v) 2-β-mercaptoethanol, and 1 μM microcystin LR. Reactions were initiated by the addition of substrate. MAPK reactions were terminated by transfer to p81 filter paper; the filters were rinsed repeatedly in 185 mM orthophosphoric acid and then dehydrated in acetone. JNK assays were terminated by transfer to 10% polyacrylamide gels; phosphorylated products were resolved by electrophoresis, and appropriate substrate bands were excised. Total radioactivity in gels and filters was determined by liquid scintillometry.

Statistical Analysis. The significance of differences between experimental conditions was determined using Student's *t* test for unpaired observations.

RESULTS

In initial studies, the dose-response of empty vector and p21 antisense-expressing cell lines to ara-C-mediated apoptosis was examined (Fig. 1). After a 6-h incubation with increasing concentrations of ara-C, empty vector transfectants (pREP4) displayed a progressive increase in the percentage of cells displaying the characteristic morphological features of apoptosis, reaching a maximum of ~30% at 100 μM (Fig. 1*A*). This response was similar to that observed in untransfected cells (not shown). Both antisense-expressing cell lines (p21AS/F4 and/B8) exhibited a significantly greater susceptibility to apoptosis at each ara-C concentration evaluated. Gel electrophoretic analysis of DNA obtained from cells treated with various concentrations of ara-C also revealed more intensely staining oligonucleosomal bands in p21AS/F4 cells compared with empty vector controls (Fig. 1*B*). In addition, degradation of the M_r 115,000 caspase-3 substrate PARP into its M_r 85,000 cleavage product was considerably more prominent in p21AS/F4 and/B8 cells than in their empty vector counterparts (Fig. 1*C*). As reported previously in the case of p21 antisense-expressing HL-60 cells (15), a 6-h exposure to ara-C did not result in a discernible increase in $p21^{WAF1/CIP1}$ expression (Fig. 1*D*). Taken together, these findings demonstrate that the presence of the p21 antisense construct rendered U937 leukemic cells more sensitive to ara-C-mediated apoptosis.

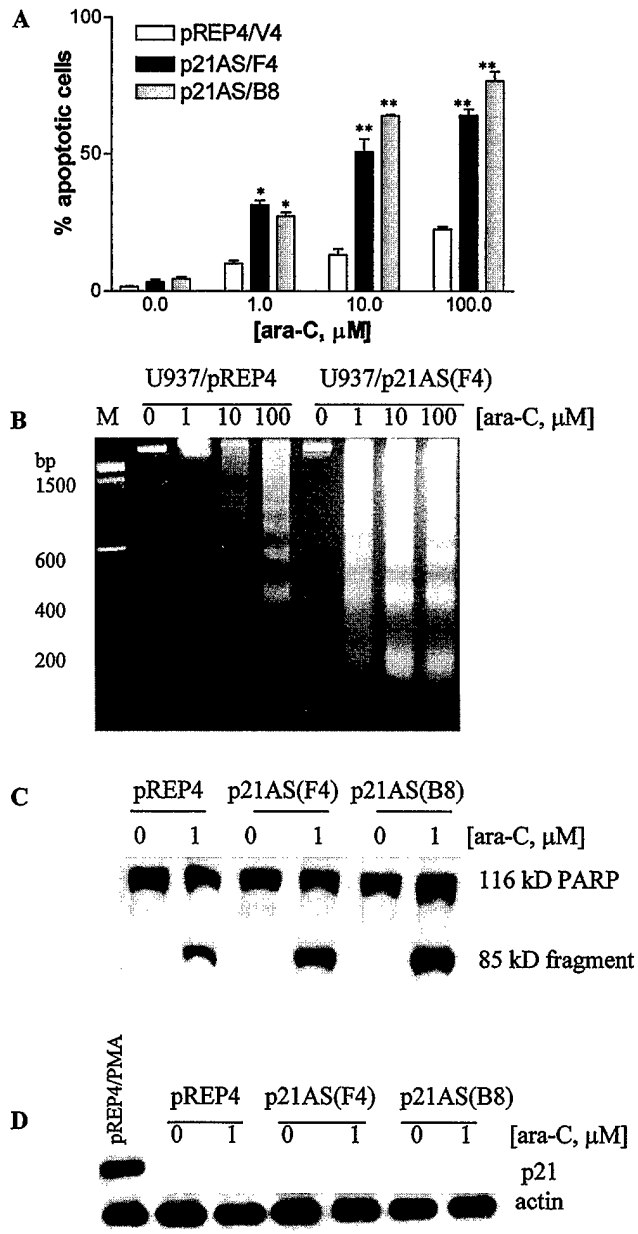

Fig. 1. *A*, cells containing an empty vector (pREP/V4) and two antisense-expressing clones (p21AS/F4 and B8) were exposed to the designated concentration of ara-C for 6 h, after which the percentage of apoptotic cells was determined by evaluating Wright Geimsa-stained specimens as described in the text. Values represent the means for three separate experiments; *bars*, $\pm$1 SD. *, greater than values for empty vector controls; $P \leq 0.05$; **, $P \leq 0.02$. *B*, DNA was extracted from empty vector control and antisense-expressing cells (AS/F4) after a 6-h exposure to the designated concentration of ara-C, subjected to agarose gel electrophoresis, and stained with propidium iodide to visualize internucleosomal DNA cleavage patterns. A standard DNA ladder is shown in the left lane. *C*, after exposure to 1 μM ara-C for 6 h, cells were lysed, protein was extracted, and expression of PARP (20 μg/condition) was assessed by Western analysis as described in the text. Proteolysis of native M_r 116,000 PARP to its M_r 85,000 cleavage product characteristically occurs in cells undergoing apoptosis. *D*, Western analysis of $p21^{WAF1/CIP1}$ expression after exposure of cells to 1 μM ara-C for 6 h. An extract from pREP4 cells treated with 10 nM PMA for 24 h was used as a positive control.

Because the susceptibility of neoplastic cells to diverse chemotherapeutic agents can depend upon the relative abundance of pro- and antiapoptotic members of the Bcl-2 family (24), Western blot analysis was performed to determine if such factors might be responsible for the preceding observations. However, levels of Bcl-2, Bcl-x_L, and Bax

were equivalent in untransfected cells, cells transfected with the empty vector, and in the two p21 antisense-expressing cell lines (data not shown). Furthermore, treatment with 1 μM ara-C for 6 h failed to modify expression of these proteins, rendering it unlikely that alterations in levels of Bcl-2, Bcl-x_L, or Bax could account for the increased sensitivity of the p21antisense-expressing lines to ara-C-induced apoptosis. The cytotoxic actions of ara-C have also been related to formation of its lethal triphosphate derivative, ara-CTP, and/or to incorporation of ara-C residues into elongating DNA strands (25). However, after a 6-h exposure to 1 μM ara-C, both ara-CTP formation (27.6 ± 3.5 *versus* 31.3 ± 3.3 pmol ara-CTP/10^6 cells) and ara-C(DNA) incorporation (1.21 ± 0.23 *versus* 1.51 ± 0.41 pmol/ng DNA) were equivalent in the pREP4 and p21AS/F4 cell lines ($P \leq 0.05$ in each case; data not shown).

It remained possible that the increased susceptibility of p21 antisense-expressing cells to ara-C might stem from cytokinetic or pharmacodynamic factors. For example, because ara-C is an S-phase-specific agent (26), an increase in the S-phase fraction would render cells more vulnerable to this agent. However, flow cytometric analysis revealed that the S-phase percentage of the pREP4 and p21AS/F4 lines were identical (*e.g.,* 38.6 ± 3.6 *versus* 40.8 ± 4.2; $P \geq 0.05$; Fig. 2*A*). Moreover, the sub-G_1 population, corresponding to hypodiploid, apoptotic cells, was clearly greater in p21 antisense-expressing cells after ara-C administration (Fig. 2*B*). Furthermore, the increase in the subdiploid population was observed over a wide range of ara-C concentrations (Fig. 2*C*). Together, these and the preceding findings indicated that the increased susceptibility of p21 antisense-expressing cells to ara-C-mediated apoptosis could not simply be attributed to conventional cytokinetic or pharmacodynamic factors.

It has been shown previously that ara-C-induced apoptosis in human leukemia cells (HL-60) is accompanied by dephosphorylation of the retinoblastoma protein (27). To assess the impact of $p21^{WAF1/CIP1}$ dysregulation on this phenomenon, pRb phosphorylation status was monitored in U937pREP4 and U937p21AS/F4 cells after a 6-h exposure to 1 μM ara-C (Fig. 3). U937/pREP4 cells displayed a marginal increase in abundance of the higher mobility pRb species after treatment with 1 μM ara-C for 6 h (Fig. 3*A*). However, this shift was considerably more pronounced in the U937/p21AS/F4 line, a finding that is distinctly different from that observed previously in p21 antisense-expressing cells treated with PMA (16). Results obtained using the G99-549 murine antibody, which primarily recognizes underphosphorylated pRb (28), supported the notion that $p21^{WAF1/CIP1}$ dysregulation promotes ara-C-mediated pRb dephosphorylation (Fig. 3*B*). In contrast to these findings, expression of c-Myc protein was unperturbed by ara-C treatment in both the p21 antisense-expressing and empty vector control cell lines (Fig. 3*A*).

It is now recognized that a discordance may exist between the appearance of the classic morphological and biochemical features of apoptosis and loss of cellular clonogenic potential (29, 30). To assess the biological consequences of $p21^{WAF1/CIP1}$ dysregulation, the viability of ara-C-treated empty vector and p21 antisense-expressing cells was compared using trypan blue exclusion and clonogenic assays (Fig. 4). After a 6-h exposure to various concentrations of ara-C, p21 antisense-expressing cells displayed a highly significant reduction in the number of viable cells at 24 h (Fig. 4*A*) and in the number of day 12 colonies (Fig. 4*B*) than their empty vector-containing counterparts. These results indicate that the increased susceptibility of p21 antisense-expressing U937cells to ara-C-mediated apoptosis is accompanied by a corresponding loss of leukemic self-renewal capacity.

Subsequent studies were undertaken to elucidate cytokinetic factors potentially involved in the increased sensitivity of p21 antisense-expressing cells. The nucleoside APH, like ara-C, is an inhibitor of DNA polymerase, but unlike ara-C, is not incorporated into elongating DNA strands (31). Although APH is known to induce apoptosis by itself (29), a minimally toxic concentration (0.1 μM) was identified that was capable of arresting ~85% of cells in S-phase after a 24-h exposure (data not shown). Cells were then washed free of APH, and the partially synchronized population was exposed to ara-C as described above. It can be seen from the data shown in Fig. 5 that pretreatment of U937/pREP4 cells with a subtoxic concentration of APH significantly increased the extent of apoptosis resulting from a

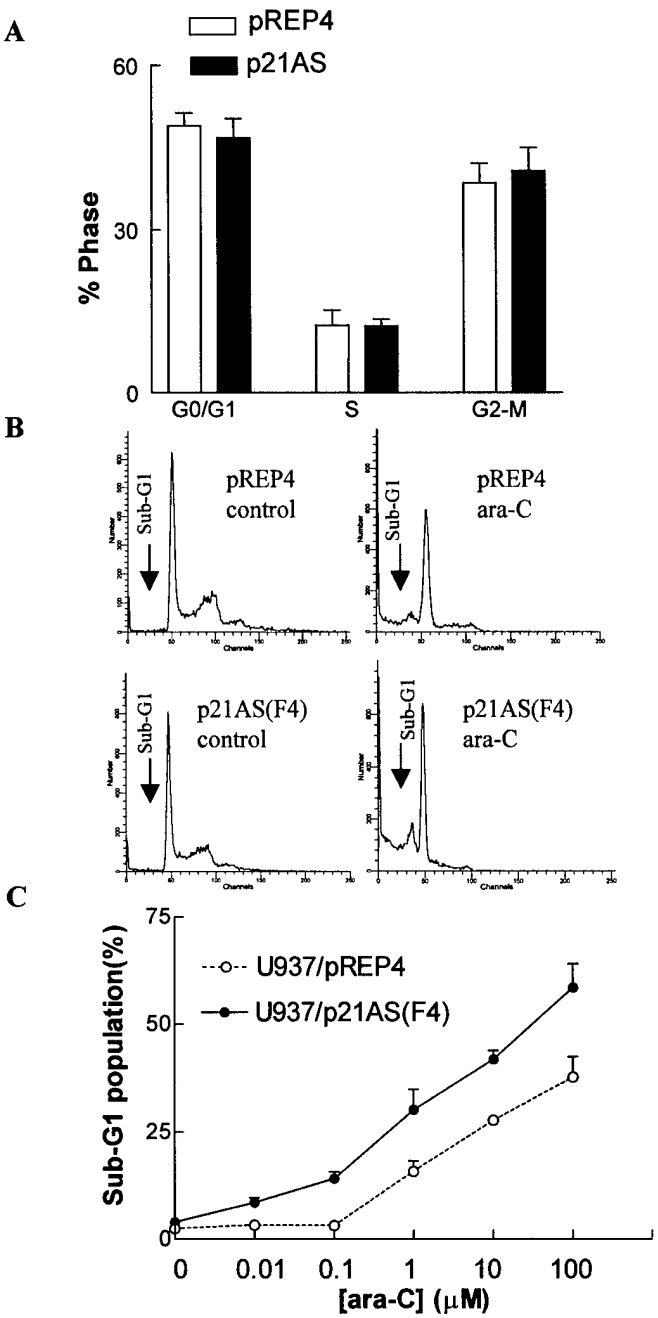

Fig. 2. *A,* cells were incubated with propidium iodide, and cell cycle characteristics were determined using a Becton Dickinson FACScan flow cytometer and ModFit software as described in "Materials and Methods." The percentage of cells in the G_0/G_1, S, and G_2-M fractions of the cell cycle represent the means for three separate experiments; *bars,* SD. *B,* after exposure to 1 μM ara-C for 6 h, cell pellets were incubated in 70% ethanol overnight at 4°C to promote loss of fragmented DNA. The cells were then treated with propidium iodide and subjected to cell cycle analysis as above. The sub-G_1 populations, corresponding to hypodiploid, apoptotic cells, are shown under the *arrows. C,* pREP4 and p21AS/F4 cells were exposed to the designated concentration of ara-C for 6 h, after which the sub-G_1 fraction was quantified by flow cytometry as above. Values represent the means for three separate experiments; *bars,* SD.

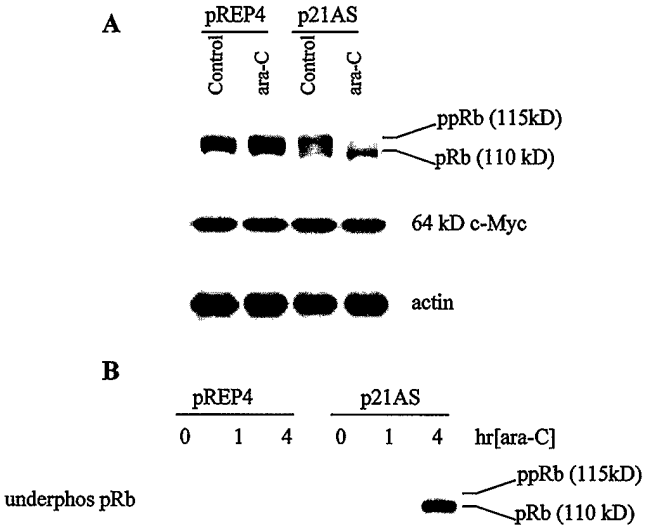

Fig. 3. *A*, pREP4 and p21AS/F4 cells were exposed to 1 μM ara-C for 6 h, after which they were lysed, and expression of pRb and c-Myc were determined by Western blot analysis as described in the text. Each lane was loaded with 10 μg of protein. Phosphorylated pRb characteristically displays a mobility of M_r 116,000, whereas dephosphorylated pRb migrates at M_r 110,000. *B*, cell lysates were obtained and analyzed as above, except that they were probed with a murine antibody that primarily recognizes underphosphorylated pRb (G99-549), as described in the text. The results of a representative study are shown; two additional experiments yielded similar results.

6-h exposure to 1 μM ara-C (Fig. 5*A*). However, a similar effect was observed in U937p21AS/F4 cells; in fact, synchronization with APH permitted this ara-C exposure to induce apoptosis in ~70% of cells (*versus* ~35% in their unsynchronized counterparts). These findings were confirmed by studies examining ara-C-mediated activation of caspase-3 (CPP32; Yama), manifested by the appearance of the M_r 17,000 cleavage product, in cells synchronized by APH pretreatment (Fig. 5*B*). The results of these studies demonstrated that cells synchronized by APH treatment (Fig. 5*B, Lanes 4 and 8*) displayed greater caspase-3 cleavage than their unsynchronized counterparts (Fig. 5*B, Lanes 3 and 7*); moreover, in each case, caspase -3 cleavage was greater in antisense-expressing (Fig. 5*B, Lanes 7 and 8*) than in empty vector-containing cells (Fig. 5*B, Lanes 3 and 4*). Taken together, such findings suggest that loss of $p21^{WAF1/CIP1}$ function specifically increases the susceptibility of S-phase cells to ara-C-related cell death, presumably by facilitating activation of the apoptotic caspase cascade.

There is abundant evidence that mitochondrial dysfunction plays an important, and perhaps central, role in apoptotic events (17). To determine what effect $p21^{WAF1/CIP1}$ dysregulation might have on mitochrondrial perturbations accompanying (or responsible for) apoptosis, uptake of the lipophilic fluorochrome $DiOC_6$ was monitored (Fig. 6*A*). Loss of mitochondrial membrane potential ($\Delta\psi_m$) is characteristically associated with reduced cellular accumulation of $DiOC_6$ (17). After a 6-h exposure to 1 μM ara-C, a significantly greater percentage of p21 antisense-expressing cells displayed a reduction in $\Delta\psi_m$ compared with their empty vector counterparts (*e.g.*, 52.8 $\pm$ 3.0% *versus* 30.2 $\pm$ 2.8%; $P \leq 0.02$; $n = 3$). Equivalent results were obtained using the fluorochrome JC-1 (data not shown). Similarly, the generation of ROS by ara-C, manifested by increased staining with DHR 123, was greater in antisense-expressing than in empty vector-containing cells (*e.g.*, 18.7 $\pm$ 1.5% *versus* 5.5 $\pm$ 1.7%; $P \leq 0.02$; $n = 3$; Fig. 6*B*). However, the percentage of cells positive for ROS was less than the percentage of cells displaying loss of $\Delta\psi_m$ (or characteristic apoptotic morphology), suggesting that ROS generation represents a secondary event in this setting. Lastly, ara-C-mediated increases in release of cytochrome *c* into the cytosolic S-100 fraction were enhanced in p21 antisense-expressing cells (relative to empty-vector controls) as early as 2 h after drug exposure and was quite marked at 4–6 h (Fig. 6*C*). Collectively, these findings indicate that $p21^{WAF1/CIP1}$ dysregulation is accompanied by potentiation of mitochondrial dysfunction in ara-C-treated cells, and that this phenomenon represents an early event in the cell death program.

Finally, it has been shown that cellular susceptibility to apoptosis may depend upon the balance between pro- (*e.g.*, JNK/SAPK) *versus* anti- (*e.g.*, ERK/MAPK) apoptotic signaling pathways (32). Furthermore, links between $p21^{WAF1/CIP1}$ and these pathways have been identified, inasmuch as MAP kinase has been shown to be involved in regulation of $p21^{WAF1/CIP1}$ expression (33), and p21 has been reported to inhibit JNK activation (34). Consequently, the effect of $p21^{WAF1/CIP1}$ dysregulation was examined with respect to its effects on ara-C-mediated MAP kinase and JNK activation. Basal JNK activity was equivalent in empty vector controls and the two antisense-expressing lines (*e.g.*, 3.7 $\pm$ 1.2, 3.5 $\pm$ 1.0, and 3.1 $\pm$ 0.6 fmol/min/mg protein; data not shown). In addition, no differences in JNK activation were observed between the cell lines after ara-C exposure. In contrast to these findings, basal MAP kinase activity was significantly higher in p21 AS-expressing cells than in empty vector controls (*e.g.*, 286 $\pm$ 71 and 373 $\pm$ 112 *versus* 103 $\pm$ 26 fmol/min/mg for p21AS/F4, p21AS/B8, and pREP4, respectively; $P \leq 0.05$ in each case; Fig. 7*A*). Furthermore, upon ara-C exposure, pREP4 cells displayed increases

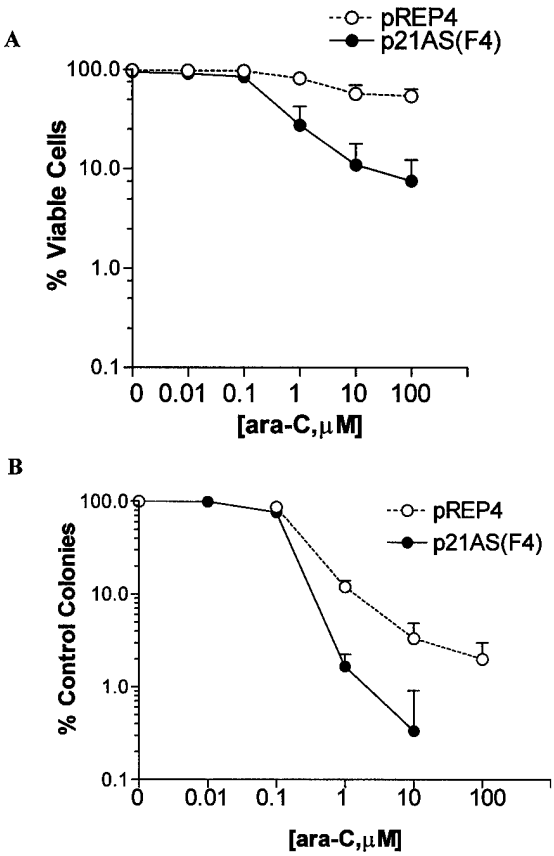

Fig. 4. *A*, pREP4 and p21AS/F4 cells were exposed to the designated concentration of ara-C for 6 h, washed, and resuspended in drug-free medium for an additional 24 h. The percentage of viable cells (relative to untreated controls) was determined by monitoring trypan blue exclusion as described in the text. Values represent the means for three separate experiments; *bars*, SD. *B*, cells were exposed to the designated concentration for 6 h, washed free of drug, and plated in soft agar as described in "Materials and Methods." Colonies, consisting of groups of $\geq$50 cells, were scored on day 12. Values are expressed as the percentage of colonies formed relative to untreated controls and represent the means for three separate experiments; *bars*, SD.

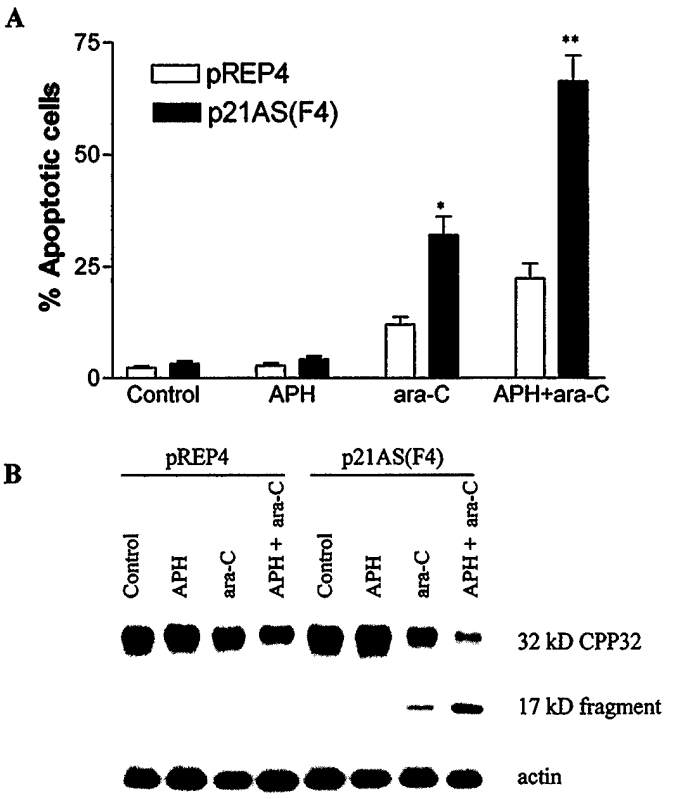

Fig. 5. *A*, pREP4 and p21AS/F4 cells were incubated for 24 h with 0.125 μM APH, after which they were washed free of drug and resuspended in complete medium. After such treatment, 75–85% of the cells accumulated in S-phase. The cells were then exposed to 1 μM ara-C for 6 h, after which the percentage of apoptotic cells was determined by examining Wright Giemsa-stained preparations. Values represent the means for three separate experiments; *bars*, SD. *, significantly greater than values for pREP4 cells: $P \leq 0.02$; **, $P \leq 0.005$. *B*, after treatment as above, expression of the M_r 32,000 Yama protein and its M_r 17,000 cleavage product were monitored by Western blot analysis as described in "Materials and Methods." Each lane was loaded with 10 μg of protein. *Lanes 1–4*, pREP4 cells; *Lanes 5–8*, p21AS/F4 cells. *Lanes 1* and *5*, controls; *Lanes 2* and *6*, APH (0.125 μM; 24 h); *Lanes 3* and *7*, ara-C (1 μM; 6 h); *Lanes 4* and *8*, APH → ara-C. Two additional studies yielded equivalent results.

in MAP kinase activity after 3–4 h, followed by a return toward basal levels (Fig. 7*A*). In contrast, antisense-expressing cells expressed a rapid (*e.g.*, within 15 min) reduction in MAP kinase activity, followed by a partial recovery and subsequent decline (Fig. 7*A*). At no time did antisense-expressing cells exhibit an increase in MAP kinase activity over basal levels. Changes in MAP kinase activity (relative to initial values) are shown more clearly in Fig. 7*B*. However, because basal MAP kinase activity in pREP4 cells was significantly less than that observed in the antisense-expressing lines, absolute levels of activity in the former cells were only slightly greater than (or equivalent to) those detected in the p21AS/F4 and B8 cells 4–6 h after ara-C exposure. Finally, treatment with 100 nM PMA increased MAP kinase activity (at 20 min) by ≥100% in each of the cell lines (data not shown), indicating that constitutive activity was not maximally stimulated in antisense-expressing cells. These findings raise the possibility that the failure of ara-C to activate the cytoprotective MAP kinase signaling pathway over basal levels in $p21^{WAF1/CIP1}$ antisense-expressing cells may contribute to their increased susceptibility to ara-C-mediated apoptosis.

DISCUSSION

The CDKI $p21^{WAF1/CIP1}$, a downstream target of p53, has been implicated in the G_1 arrest response to both DNA-damaging and differentiation-inducing agents (5, 6, 35). Furthermore, evidence that induction of $p21^{WAF1/CIP1}$ in differentiating myocytes is associated with a reduced susceptibility to cell death (36) argues for an antiapoptotic role for this CDKI. Moreover, human colon tumor cells (HCT116) deficient in $p21^{WAF1/CIP1}$ undergo apoptosis more readily than their wild-type counterparts in response to agents such as doxorubicin and ionizing radiation, a phenomenon attributed to uncoupling of S-phase and mitosis (8, 13). More recently, inducible up-regulation of $p21^{WAF1/CIP1}$ was shown to promote DNA repair in human glioblastoma cells (LN-Z308 and U251) and render them less susceptible to alkylating agent-mediated apoptosis (10). Together, these findings suggest that $p21^{WAF1/CIP1}$ protects cells from drug-mediated lethality through cell cycle- and/or DNA repair-related mechanisms.

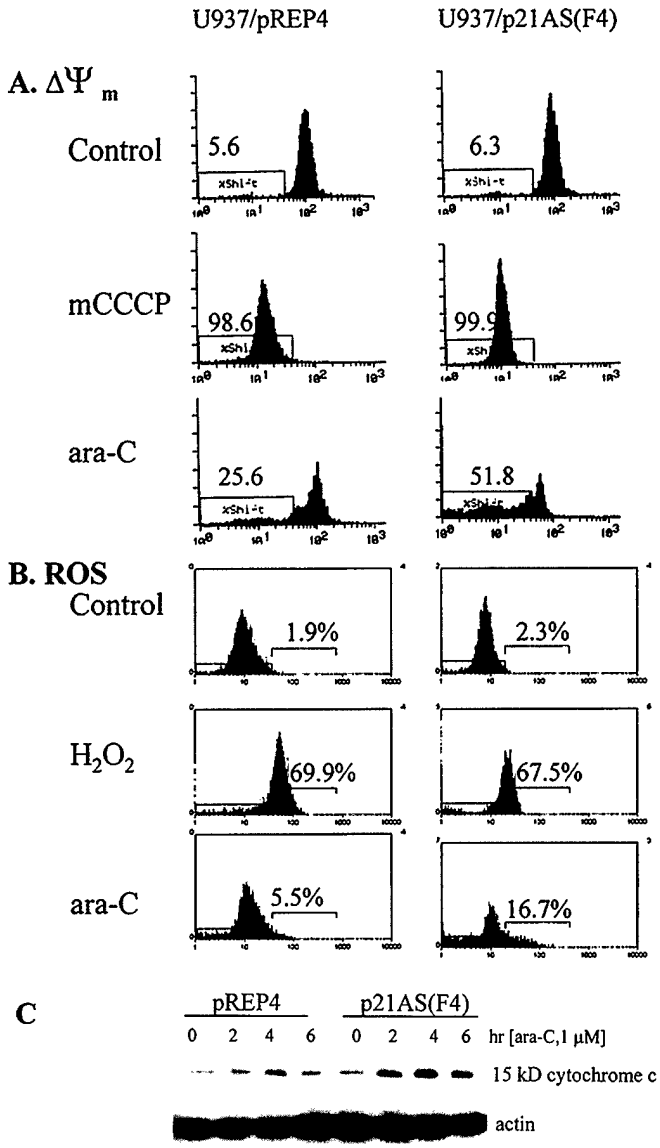

Fig. 6. *A*, after exposure of pREP 4 and p21AS/F4 cells to 1 μM ara-C for 6 h, $\Delta\psi_m$ was monitored by flow cytometry using $DiOC_6$ as a probe. Results obtained with the protonophore *m*-chlorophenylhydrazone (*mCCCP*), which causes complete collapse of the $\Delta\psi_m$, are shown for comparison. Values represent the percentage of cells exhibiting low $DiOC_6$ uptake; two additional studies yielded equivalent results. *B*, generation of ROS was monitored in cells treated as above by flow cytometry using DHR 123 as described in the text. Results obtained using H_2O_2 are shown for comparison. Values represent the percentage of cells exhibiting high DHR 123 fluorescence; two additional studies yielded equivalent results. *C*, after treatment as above, cytosolic S-100 fractions were isolated from pREP4 and p21AS/F4 cells, and expression of cytochrome *c* was assessed by Western blot analysis as described in the text. Actin controls are shown to document equal loading of lanes and protein transfer. Two additional studies yielded equivalent results.

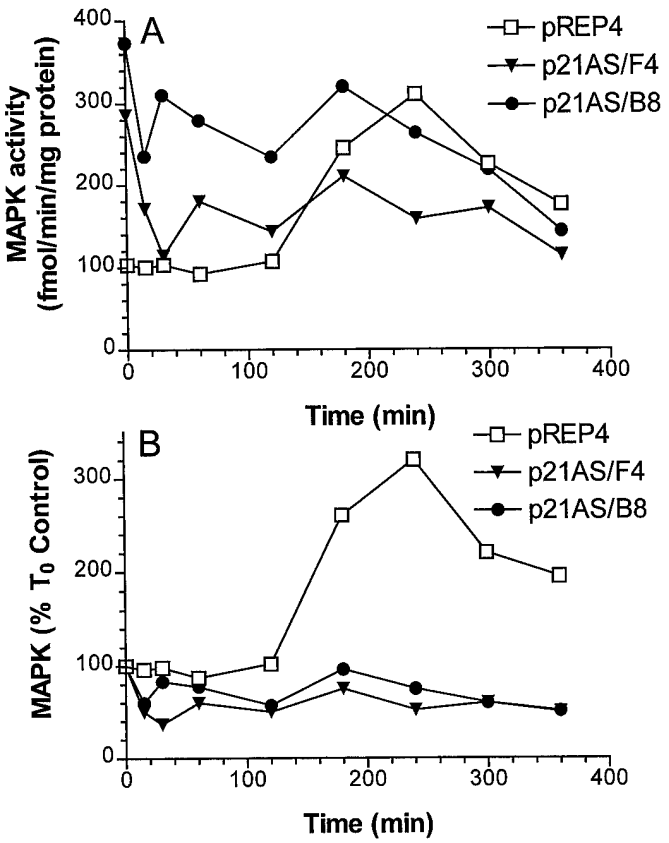

Fig. 7. *A*, logarithmically growing pREP4 (□), p21AS/F4 (▼), and p21AS/B8 (●) cells were exposed to 1 μM ara-C for 6 h, and at the designated intervals, MAP kinase activity, manifested by the phosphorylation of myelin basic protein, was determined as described in "Materials and Methods." Values, expressed as fmol [^{32}P]myelin basic protein/min/mg protein, represent the means for duplicate experiments. *B*, MAP kinase activity was monitored in ara-C-treated cells as described above and at each interval was expressed as a percentage relative to T_0 control values. In each case, variability between experiments was generally ≤15%.

Despite certain similarities, important differences exist between the present results and results reported previously. For example, in the study by Waldman *et al.* (8), enhanced apoptotic responses of p21$^{WAF1/CIP1}$-deficient cells to DNA-damaging agents were noted at relatively late time intervals (*e.g.*, at 30 h and most notably at 60–90 h after drug administration), which permitted cells to undergo at least one and in some cases more than one round of DNA synthesis. Analogously, in the study by Ruan *et al.* (10), p21$^{WAF1/CIP1}$-mediated protection from alkylator-induced apoptosis was noted 7 days after drug administration. In marked contrast, dysregulation of p21$^{WAF1/CIP1}$ in U937 cells rendered them dramatically more susceptible to ara-C-induced apoptosis within 4–6 h of drug exposure. In view of the relatively long generation time of leukemic blasts (*e.g.*, ≥ 30 h; 37), it is extremely unlikely that a shortened cell cycle traverse of p21 antisense-expressing cells could fully account for the early increase in drug susceptibility. Furthermore, partial synchronization of cells in S-phase by APH amplified the differential sensitivity of control and mutant cells to ara-C-induced cell death, indicating that dysregulation of p21$^{WAF1/CIP1}$ enhanced the intrinsic vulnerability of S-phase cells to ara-C actions. Although interference with DNA repair by p21$^{WAF1/CIP1}$ dysregulation cannot be ruled out, equivalent incorporation of ara-C residues into the DNA of mutant and control cells argues against this possibility. Collectively, these findings suggest that the mechanism by which p21$^{WAF1/CIP1}$ dysregulation renders U937 cells more sensitive to ara-C at early time points differs fundamentally from that involved in late-stage potentiation of apoptosis in colon tumor and glioblastoma cells treated with DNA-damaging agents (8–10).

Given evidence linking apoptosis to mitochondrial events (38, 39), it is tempting to relate increased ara-C susceptibility of p21$^{WAF1/CIP1}$ antisense-expressing cells to a lowered threshold for mitochondrial damage. Release of cytochrome *c* from mitochondria leads to the formation, in the presence of dATP, of a complex consisting of apoptosis-activating factor 1 (Apaf-1; the mammalian *Ced*-4 homologue) and caspase-9 (Apaf-3; 40), which, in turn, cleaves and activates caspase-3 (Yama; CPP32), thereby initiating the proteolytic apoptotic cascade. It is presently uncertain whether loss of mitochondrial membrane potential represents the central initiating event in apoptosis (17), given evidence that cytochrome *c* release may precede the collapse in $\Delta\psi_m$ (18, 41). On the other hand, the ability of certain agents to induce apoptosis in the absence of cytochrome *c* release (42, 43) suggests the existence of cytochrome *c*-independent cell death pathways. In the present study, the reduction in $\Delta\psi_m$ and release of cytochrome *c* accompanying ara-C treatment was more marked in p21-antisense cells as early as 2 h and most prominently at 4–6 h after drug exposure, although the close temporal relationship between these events makes it difficult to determine which was primarily responsible for enhanced lethality. Nevertheless, these findings indicate that dysregulation of p21$^{WAF1/CIP1}$ lowers the threshold for mitochondrial dysfunction after ara-C treatment. It is noteworthy that p21 antisense-expressing cells exposed to ara-C displayed a relatively modest enhancement in ROS generation, consistent with the notion that induction of ROS lags behind and represents a consequence of earlier mitochondrial damage (41). It is also important to note that the increased susceptibility of p21$^{WAF1/CIP1}$ antisense-expressing cells to ara-C-mediated mitochondrial dysfunction was accompanied by a loss in leukemic cell self-renewal capacity, particularly in view of reports suggesting a discordance between apoptosis and loss of clonogenic potential (27, 30, 44).

Although disruption of p21$^{WAF1/CIP1}$ function has been shown to increase the sensitivity of tumor cells to various agents (8–10), this has not been a universal finding. For example, antisense-mediated disruption of p21$^{WAF1/CIP1}$ reduced antioxidant-related apoptosis and potentiation of 5-FU-mediated lethality in human colon cancer cells (HCT 15 and HCT 116) in a p53-independent manner (45). However, interference with p21$^{WAF1/CIP1}$ function in HCT 116 cells did not directly alter 5-FU sensitivity. Enforced expression of p21$^{WAF1/CIP1}$ has also been shown to enhance apoptosis in pRb-negative osteosarcoma cells (SaOs-2) exposed to the antimetabolites methotrexate and tomudex, a phenomenon attributed to inhibition of E2F-1 phosphorylation and activity (46). The basis for divergent effects of p21$^{WAF1/CIP1}$ dysfunction on the drug sensitivity of myelomonocytic *versus* promyelocytic leukemia cells (15) is unclear but may stem from unique features of the HL-60 cell line, including amplification of c-*myc* (47). Together, these findings indicate that the net effect of p21$^{WAF1/CIP1}$ dysregulation on drug sensitivity depends upon the molecular context in which it occurs, as well as the inciting agent.

After ara-C treatment, p21 antisense-expressing cells displayed a more prominent high mobility, putatively dephosphorylated pRb species than their empty vector counterparts. The ability of ara-C to induce pRb dephosphorylation in HL-60 cells has been described previously (27). However, it has been shown recently that cleavage of pRb during apoptosis can generate a fragment that comigrates with the dephosphorylated pRb protein (48). Although the increase in abundance of such a fragment in p21 antisense-expressing cells could represent a consequence of enhanced susceptibility to ara-C-mediated cell death, results obtained with an antibody specific for underphosphorylated pRb (Fig. 4*B*) demonstrate that p21$^{WAF1/CIP1}$ dysregulation specifically promotes ara-C-associated pRb dephosphorylation. Lastly, the divergent effects of p21$^{WAF1/CIP1}$ dysregulation on PMA-induced (16) *versus* ara-C-induced underphosphorylation of pRb sug-

gest that these agents modify pRb phosphorylation status through different mechanisms.

Several lines of evidence, including demonstration of transcriptional activation of $p21^{WAF1/CIP1}$ expression through the MAP kinase signaling cascade (33), suggest a link between $p21^{WAF1/CIP1}$ induction and the ERK/MAP kinase survival pathway. Although $p21^{WAF1/CIP1}$ has been reported to inhibit activation of the stress-related JNK/SAPK cascade (34), $p21^{WAF1/CIP1}$ dysregulation failed to modify the JNK response to ara-C in the present study. In contrast, p21 antisense-expressing cells exhibited higher basal levels of MAP kinase activity than their pREP4 counterparts but did not exhibit increases in activity after ara-C exposure. MAP kinase has been linked to cell survival (32) and has also been implicated in cellular maturation (49), an event that under some circumstances opposes drug-induced apoptosis (50). Furthermore, stresses imposed by cytotoxic drugs such as ara-C are known to induce MAP kinase in leukemic cells (51), and agents that interrupt the MAP kinase cascade (*e.g.,* PD98059) potentiate apoptosis in cells exposed to H_2O_2 (52) as well as ara-C and Taxol (53, 54). It is therefore possible that attenuation of MAP kinase activation over basal levels in p21 antisense-expressing cells contributes to their enhanced susceptibility to ara-C-related apoptosis. An alternative possibility is that functional $p21^{WAF1/CIP1}$ may be required for MAP kinase-related cytoprotective actions.

In summary, the present studies demonstrate that dysregulation of the CDKI $p21^{WAF1/CIP1}$ increases the susceptibility of U937 myelomonocytic leukemia cells to ara-C-mediated apoptosis, a phenomenon that is associated with early alterations in mitochondrial function (*e.g.,* loss of $\Delta\psi_m$ and release of cytochrome *c* into the cytosol). Thus, in addition to disruption of cell cycle-related events (9) and interference with DNA repair (10), facilitation of mitochondrial damage represents an alternative means by which $p21^{WAF1/CIP1}$ dysregulation can promote drug-related apoptosis. The mechanism by which potentiation of mitochondrial damage occurs is unclear, although it is known that $p21^{WAF1/CIP1}$ forms a complex with cyclins, CDKs, and the DNA replication/repair-related proliferating cell nuclear antigen (55). It is possible that perturbations stemming from $p21^{WAF1/CIP1}$ dysregulation modify proliferating cell nuclear antigen activity, thereby amplifying DNA damage signals through an as yet unidentified pathway. Moreover, the results of a recent study suggest that the cell cycle checkpoint gene *p53* triggers early expression of a group of redox-related p53-inducible genes, activation of which is associated with mitochondrial degradation and ultimately, cell death (56). Because $p21^{WAF1/CIP1}$ represents a major downstream effector of p53 (5) and is also induced in p53-null cells (6, 7), the notion that $p21^{WAF1/CIP1}$ might be involved in the regulation of such redox-associated p53-inducible genes appears plausible. Further studies involving $p21^{WAF1/CIP1}$ may yield important information concerning novel molecular determinants of ara-C sensitivity in leukemia, and could also provide mechanistic insights into interactions between cytotoxic drugs and differentiation-inducing agents known to trigger CDKI expression.

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The Vitamin D_3 Analog EB 1089 Enhances the Response of Human Breast Tumor Cells to Radiation

S. Sundaram and D. A. Gewirtz

Department of Pharmacology, Toxicology and Medicine, Virginia Commonwealth University—Medical College of Virginia,
P.O. Box 980230 *Richmond, Virginia 23298*

Sundaram, S. and Gewirtz, D. A. The Vitamin D_3 Analog EB 1089 Enhances the Response of Human Breast Tumor Cells to Radiation. *Radiat. Res.* 151, 000–000 (1999).

Previous studies from this laboratory as well as others have demonstrated that ~~cells of~~ breast tumor cells~~lines~~ fail to undergo ~~primary~~ apoptosis in response to agents which induce DNA damage such as ionizing radiation and the topoisomerase inhibitor adriamycin. Similarly, the primary response of breast tumor cells to vitamin D_3 [1,25-$(OH)_2$-D_3] and its analogs such as EB 1089 is growth inhibition, with apoptosis occurring in only a small fraction of the cell population. The possibility that the combination of vitamin D_3 compounds with radiation might promote cell death (i.e. through a differentiation stimulus plus DNA damage) was investigated by exposing both TP53 (formerly known as p53) wild-type and TP53 mutated breast tumor cells to 1,25-$(OH)_2$-D_3 or EB 1089 for 48 h prior to irradiation. This combination resulted in enhanced antiproliferative effects in the TP53 wild-type MCF-7 cells based on both a clonogenic assay and the determination of numbers of viable cells. The combination of EB 1089 with radiation increased DNA fragmentation based on both the terminal transferase end labeling (TUNEL) and bisbenzamide spectrofluorometric assays, suggesting the promotion of apoptosis. The observed increase in DNA fragmentation was not due to an enhancement of the extent of initial DNA damage induced by radiation. These findings suggest that vitamin D compounds may be useful in combination with radiation in the treatment of breast cancer. © 1999 by Radiation Research Society

INTRODUCTION

Breast cancer remains the most common malignant disease of middle-aged women in the United States. Radiation therapy plays a primary role in the control of breast cancer both in the reduction of breast tumors prior to surgery and in adjuvant therapy for the elimination of localized microtumors. However, the frequent recurrence of this disease indicates the existence of micrometastases outside the radiation field and/or a population of breast tumor cells that are resistant to radiotherapy, possibly due to their failure to undergo apoptosis.

Previous work from this laboratory as well as others supports the refractoriness of human breast tumor cells to death by apoptosis in response to treatments with agents that induce DNA damage such as topoisomerase II inhibitors and ionizing radiation (*1–5*). In contrast, tumor cells of hematopoietic or lymphatic origin as well as those of other tumor cell lines including colorectal adenoma and melanoma respond to radiation and other DNA-damaging agents by characteristic programmed cell death (apoptosis) (*6–10*). Recent clinical studies have demonstrated that the use of radiation in combination with chemotherapy is more effective than either modality alone in the elimination of localized breast tumors (*11, 12*). Leukemia cells as well as squamous cell carcinoma cells have also been shown to become sensitized to DNA-damaging agents by directing the cells on a pathway to differentiation using vitamin D or retinoids (*13–16*). As both 1,25-$(OH)_2$-D_3 and EB 1089 are known to cause differentiation in many cell types including breast tumor cells (*17–19*), we explored the possibility of enhancing the efficacy of radiation in both TP53 (formerly known as p53) wild-type and TP53 mutated breast tumor cells by pretreatment with 1,25-$(OH)_2$-D_3 or its analog EB 1089, which has been developed to circumvent the hypercalcemia associated with vitamin D_3.

MATERIALS AND METHODS

Materials

Cells of the human breast tumor cell line MCF-7 [TP53 wild-type and vitamin D receptor $(VDR)^+$] were obtained from the National Cancer Institute, Frederick, MD. Cells of the other breast tumor cell lines, MDA MB-231 (TP53 mutated and VDR^-), T47D (TP53 mutated and VDR^{++}) and ZR-75 (TP53 wild-type and VDR^+) were obtained from ATCC, Rockville, MD. Vitamin D_3 and its analog EB 1089 were provided by Dr. Lise Binderup, Leo Pharmaceuticals, Denmark. RPMI 1640 and supplements were obtained from Gibco Life Technologies, Gaithersburg, MD. Reagents used for the TUNEL assay (terminal transferase, reaction buffer and Fluorescein-dUTP) were purchased from Boehringer Mannheim, Indianapolis, IN. All other reagents used in the study were of analytical grade.

Cell Culture

All cells were grown from frozen stocks in basal RPMI 1640 medium supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, penicillin/streptomycin at 37°C under a humidified 5% CO_2 atmosphere. The medium used for T-47D and ZR-75 cells had additional supplementation

including Hepes (10 m*M*), L-glucose (2.5 g/liter), sodium pyruvate (1 m*M*), and bovine insulin (0.2 IU/ml).

All experiments were conducted using approximately 10^4 cells/cm^2 at day 0 with the use of time-equivalent and concentration-equivalent controls. The results shown are averages of two or three experiments. Statistical evaluation of quantitative data was performed by analysis of variance with a comparison of multiple means by the Fisher's/Scheffe test (*20*). Differences with $P < 0.05$ were considered significant. Duplicate measurements were performed on each sample assayed.

Trypan Blue Dye Exclusion

The temporal response of cells treated with 1,25-$(OH)_2$-D_3 or EB 1089 (100 n*M*) was assessed after 24, 48 and 96 h of incubation by determining the number of viable cells using trypan blue dye exclusion. To evaluate the combined effects of radiation and vitamin D compounds, cells were treated with 1,25-$(OH)_2$-D_3 or EB 1089 (100 n*M*) for 48 h followed by radiation (10 Gy) using a ^{137}Cs γ irradiator at a dose rate of 1.5 Gy/min. The total number of adherent viable cells in each flask was monitored 24 or 72 h after irradiation. To assess the effects of the various radiation doses, MCF-7 cells were treated with EB 1089 (100 n*M*) for 48 h, exposed to different doses of radiation (0.5 to 10 Gy), and allowed to grow for an additional 96 h. The number of viable cells was determined as described earlier.

Clonogenic Analysis

MCF-7 cells were treated with EB 1089 (100 n*M*) followed by different doses of radiation (0.5–5 Gy). Cells were trypsinized immediately after irradiation under sterile conditions and plated in triplicate in 60-mm tissue culture dishes at approximately 3000 cells for cells exposed to 0.5 to 2.5 Gy and 6000 cells for cells exposed to 5 Gy. After 10–14 days, the cells were fixed with 100% methanol, air-dried for 1–2 days, and stained with 0.1% crystal violet. Groups of 50 or more cells were counted as colonies and normalized for every 1000 cells plated before the surviving fraction was computed.

Alkaline Unwinding Assay

Bulk (single-strand) damage to DNA was determined using the alkaline unwinding procedure of Kanter and Schwartz (*21*). Cells were pretreated with 1,25-$(OH)_2$-D_3 or EB 1089 (100 n*M*) for up to 48 h and trypsinized. The cell pellet was resuspended in cold phosphate-buffered saline and irradiated on ice with 10 Gy. Bulk DNA damage was assessed immediately after radiation. In this assay, DNA cleavage is monitored based on the differential binding of a Hoechst dye to single- and double-stranded DNA. The results are expressed as *F* values, which is an indication of the percentage of double-stranded DNA in each sample where a value close to one is indicative of almost intact DNA (*21*).

DNA Fragmentation

Cellular DNA fragments in both cell lysates and medium were measured using a miniaturized adaptation of bisbenzamide spectrofluorometry. This assay, unlike the alkaline unwinding assay, was used to determine the delayed response to combining the vitamin D_3 compounds with radiation. The presence of nonsedimenting small DNA fragments (<3000 bp) in the supernatant after centrifugation at 50,000*g* at 4°C for 30 min was quantified by diluting the sample in modified TNE buffer containing Hoechst dye and by monitoring the net fluorescence in each sample (λ_{ex} = 365; λ_{em} = 460). Final DNA values were calculated based on calf thymus DNA standard as ng DNA/10^6 cells and expressed as the percentage increase over control values.

TUNEL Assay

The method of Gavrielli *et al.* (*22*) was used as an independent assessment of apoptosis. Combined cytospins containing both adherent and nonadherent cells were generated by centrifuging approximately 20,000 cells onto glass slides at 800 rpm for 10 min. The cells were fixed and

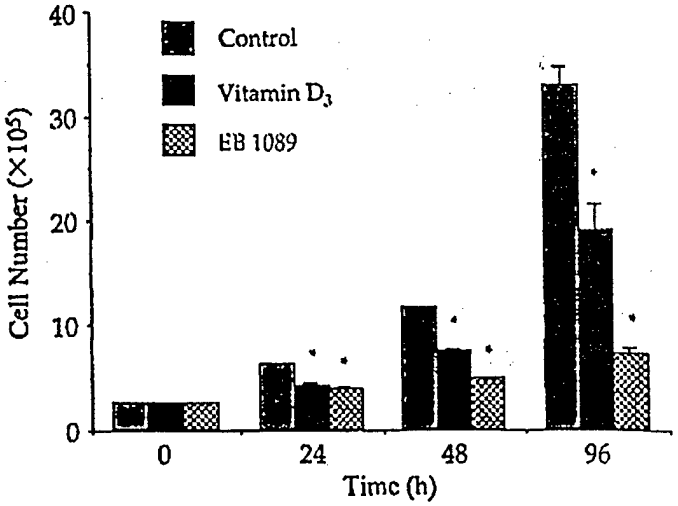

FIG. 1. Influence of 1,25-$(OH)_2$-D_3 and EB 1089 (100 n*M*) on the growth of MCF-7 cells. Cells were treated with 1,25-$(OH)_2$-D_3 and EB 1089 continuously for 24, 48 and 96 h. The final cell count was performed using trypan dye blue exclusion. Data presented are means ± SEM of two experiments. *Significantly different from their untreated control group ($P < 0.05$).

the fragmented DNA in cells undergoing apoptosis was detected using the *In Situ* Cell Death Detection Kit (Boehringer-Mannheim). In this assay, the fragmented DNA in individual cells was end-labeled using fluorescein dUTP at strand breaks by the enzyme terminal transferase as recommended by the supplier. The slides were then washed, mounted in Vectashield, and photographed using a Nikon fluorescence microscope.

RESULTS

Influence of Vitamin D_3 Compounds on the Proliferation of MCF-7 Cells

Figure 1 shows the effects of 1,25-$(OH)_2$-D_3 and EB 1089 on the number of viable MCF-7 cells after 24, 48 and 96 h of incubation. Both 1,25-$(OH)_2$-D_3 and EB 1089 (100 n*M*) showed a marked growth inhibition within 48 h of treatment, with EB 1089 being markedly more effective than the parent compound. However, at no time during the incubation with EB 1069 did the number of cells decrease below the number of cells at the initiation of the experiment with either 1,25-$(OH)_2$-D_3 or EB 1089 even with continuous exposure for up to 96 h, indicating that the primary effect of these compounds was growth inhibition (although some cell death may have occurred during the course of the study). Since a concentration of EB 1089 of 100 n*M* was effective in inhibiting cell growth within 48 h, this concentration and incubation time were used for all subsequent studies.

Potentiation of Radiation Effects by EB 1089

The effects of combining vitamin D_3 or EB 1089 with radiation were assessed in both TP53 wild-type and TP53 mutated human breast tumor cells. The cells were treated with 100 n*M* 1,25-$(OH)_2$-D_3 or EB 1089 for 48 h prior to irradiation (10 Gy). Figure 2 indicates that pretreatment o

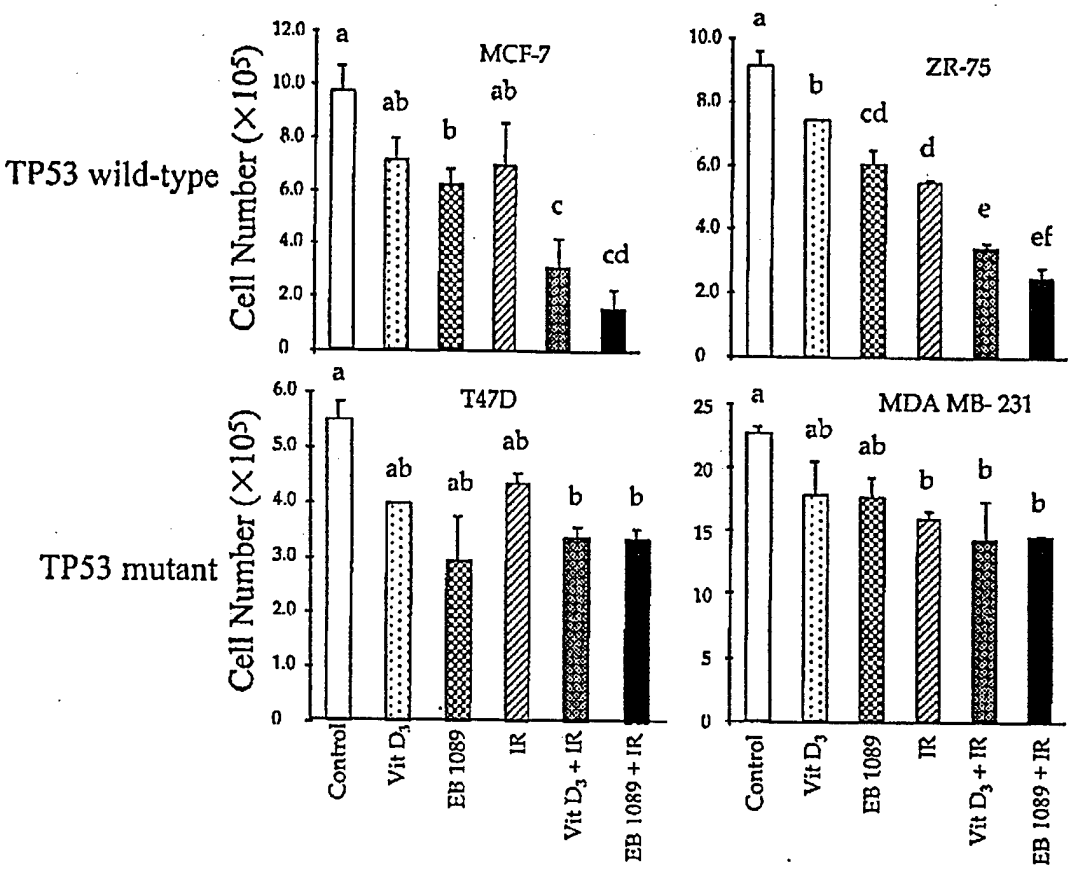

FIG. 2. Impact of pretreatment with 1,25-$(OH)_2$-D_3 or EB 1089 (100 n*M*) on the antiproliferative effects of radiation (IR) in human breast tumor cells (MCF-7, ZR-75, T47D and MDA MB-231). Cells were treated with 1,25-$(OH)_2$-D_3 and EB 1089 for 48 h, and the medium was replaced with fresh medium prior to irradiation (10 Gy). Cells were then allowed to grow at 37°C for an additional 24 h in the case of MCF-7, T47D and MDA MB-231 cells and 72 h in the case of ZR-75 cells before cell counts were made. Data presented are means ± SEM of two experiments. Bars not sharing common letter differ significantly ($P < 0.05$).

MCF-7 cells with EB 1089 prior to irradiation resulted in an approximately 90% reduction in cell number 24 h after irradiation; the combination of 1,25-$(OH)_2$-D_3 with radiation resulted in an approximately 70% reduction in cell number. In contrast, radiation, 1,25-$(OH)_2$-D_3, or EB 1089 alone inhibited cell growth by only 30%. A similar effect on the final number of cells was evident by combining radiation with 1,25-$(OH)_2$-D_3 or EB 1089 in the ZR-75 breast tumor cells which, like MCF-7 cells, are positive for the VDR (*23*) and have wild-type TP53 (*24*). Effects in ZR-75 cells were not evident until 72 h after irradiation, which is likely to be related to the slow rate of growth of this cell line. Figure 2 also shows that cells of the two TP53-mutated cell lines, T47D (*25*), which are VDR^+ (*23*), and MDA MB-231 (*24*), which are VDR^- (*23*), failed to show an enhanced response to the combined treatment with 1,25-$(OH)_2$-D_3 or EB 1089 and radiation. Final cell numbers in the groups treated with 1,25-$(OH)_2$-D_3 or EB 1089 followed by radiation did not differ significantly from those for treatment with radiation, 1,25-$(OH)_2$-D_3 or EB 1089 alone for both of these TP53-mutated cell lines.

Indications of Apoptosis in MCF-7 Cells after Combined Treatment with EB 1089 and Radiation

One of the hallmarks of death by apoptosis is the induction of DNA fragmentation. Therefore, the amount of low-molecular-weight DNA generated in cells exposed to the vitamin D_3 compounds in combination with radiation was assessed 24 h after irradiation to determine whether delayed apoptosis was occurring. The results shown in Fig. 3 indicate that 1,25-$(OH)_2$-D_3 or EB 1089 alone failed to cause any significant DNA fragmentation, while radiation produced a low baseline level of fragmentation in MCF-7 cells. The combination of 1,25-$(OH)_2$-D_3 or EB 1089 with radiation resulted in a significant increase (approximately fourfold) in the amount of fragmented DNA over that observed with radiation alone. While the combination of 1,25-$(OH)_2$-D_3 with radiation appeared to increase the extent of DNA fragmentation, this effect could not be shown to be statistically significant. These findings suggest that, in contrast to growth arrest which occurs in the 24–72 h after irradiation, 1,25-$(OH)_2$-D_3 or EB 1089 alone, cell death by

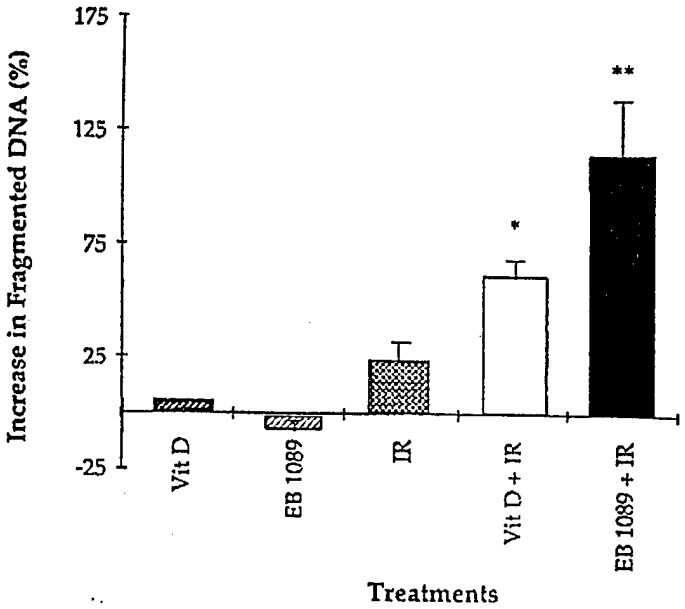

FIG. 3. Spectrofluorometric analysis of DNA fragmentation in MCF-7 cells. Cells were treated with 1,25-$(OH)_2$-D_3 or EB 1089 (100 n*M*) for 48 h followed by irradiation (IR; 10 Gy). The amount of fragmented DNA was quantified 24 h after irradiation. Data represent means ± SEM of three experiments. *Significantly different from vitamin D treatment group. **Significantly different from EB 1089-alone and radiation-alone treatment groups ($P < 0.05$).

apoptosis (or necrosis) may occur when MCF-7 cells ar treated with 1,25-$(OH)_2$-D_3 or EB 1089 prior to irradiatior

The capacity of EB 1089 to promote DNA fragmentatio in irradiated cells was demonstrated further using the TU NEL assay (Fig. 4). The amount of fluorescence in indivic ual cells treated with EB 1089 prior to irradiation wa greater than that in control cells or in cells treated wit either EB 1089 or radiation alone. This increase in fluores cence was seen throughout the cell rather than being lo calized in the nucleus. A less intense fluorescence was als observed with 1,25-$(OH)_2$-D_3 combined with radiation (nc shown), which would be consistent with the relative in creases in DNA fragmentation induced by 1,25-$(OH)_2$-D and EB 1089 in combination with radiation presente Fig. 3.

Influence of EB 1089 on Bulk DNA Damage Caused by Radiation

To rule out the possibility that the cells treated with 1,25 $(OH)_2$-D_3 or EB 1089 prior to radiation might be increasin the extent of initial DNA damage induced by radiation, w evaluated the amount of initial bulk DNA damage cause immediately after irradiation using the alkaline unwindin assay. The results in Fig. 5 indicate that, as expected, 1,25 $(OH)_2$-D_3 or EB 1089 failed to induce any DNA damage while radiation induced detectable strand breaks, reducin the double-stranded DNA by approximately 30%. Pretreat

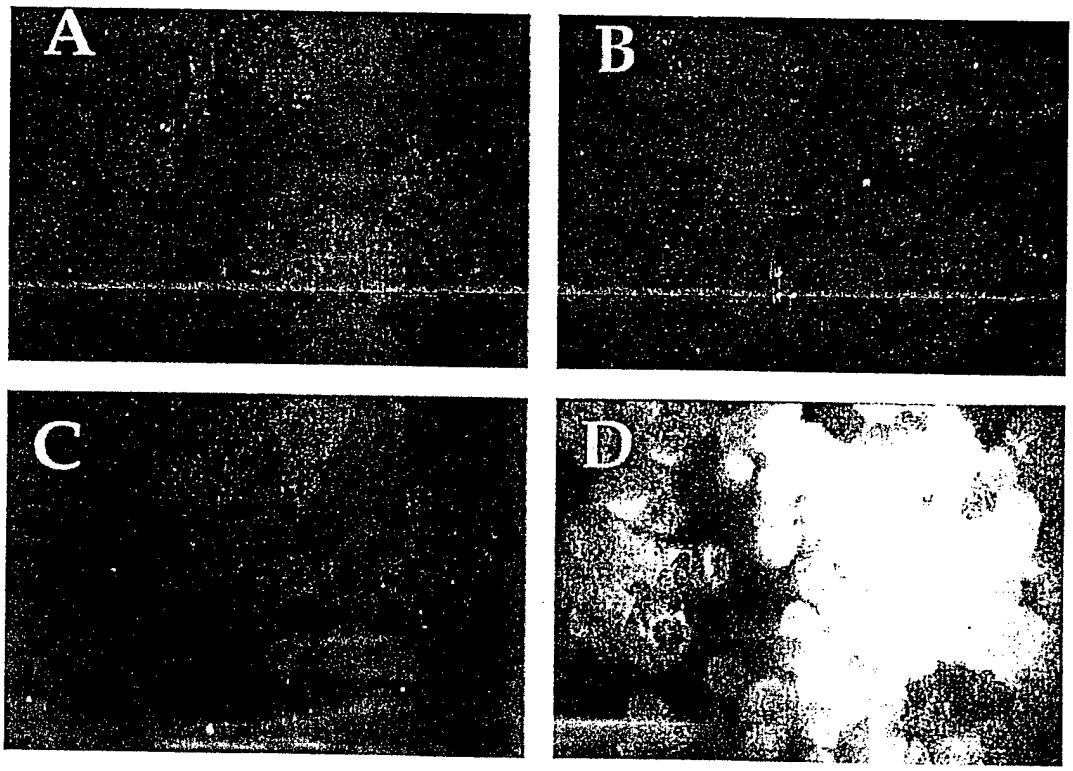

FIG. 4. Effects of combining ~~1,25-(OH)$_2$-D$_3$ or~~ EB 1089 with radiation (10 Gy) in inducing DNA fragmentation in MCF-7 cells as determined by fluorescence end-labeling. Cells were isolated 24 h after irradiation, cytospun onto glass slides, and stained according to the TUNEL protocol (see the Materials and Methods). Panel A: Control; panel B: EB 1089 (100 n*M*); panel C: radiation (10 Gy); panel D: EB 1089 + radiation. Original magnification 150×.

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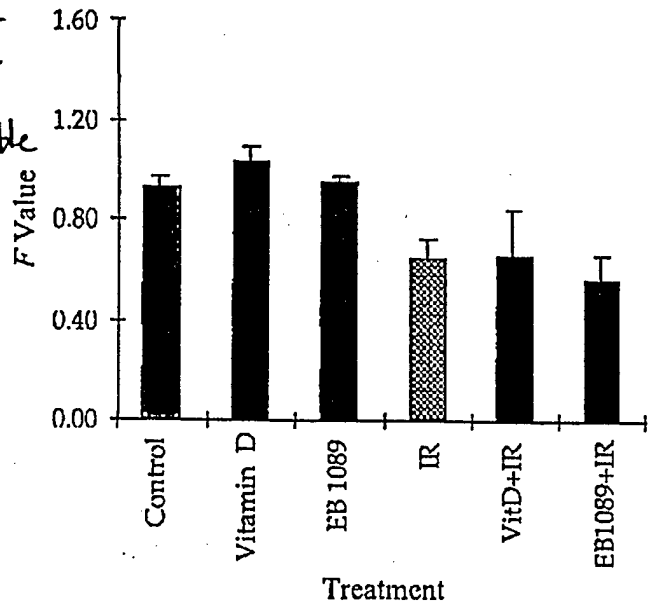

FIG. 5. Effects of pretreatment of MCF-7 cells with 1,25-$(OH)_2$-D_3 or EB 1089 on the initial DNA damage induced by radiation (IR; 10 Gy). After treatment with 1,25-$(OH)_2$-D_3 or EB 1089, MCF-7 cells were harvested and irradiated on ice. DNA damage was assessed immediately after irradiation using the alkaline unwinding assay. Data represent means ± SEM of three experiments.

ment with either 1,25-$(OH)_2$-D_3 or EB 1089 did not cause a significant increase in the initial DNA damage caused by radiation, indicating that the enhancement of cell killing observed is not due simply to increased strand breaks.

To determine how the combination of EB 1089 might influence the survival of breast tumor cells in response to radiation, MCF-7 cells were exposed to different doses of radiation with and without EB 1089 pretreatment. Studies were performed both by determining viable cell numbers 96 h after treatment (Fig. 6A) and by a clonogenic assay (Fig. 6B). The shift in the dose–response curve in Fig. 6A indicates that the dose of radiation required to produce a 70% reduction in the number of viable cells 96 h after irradiation is between 2- and 2.8-fold higher in the absence of EB 1089 than with prior exposure to EB 1089. The shift in the dose–response curve in Fig. 6B indicates that the dose of radiation required to produce a 50% reduction in clonogenicity is about 4-fold higher in the absence of EB 1089 than with prior exposure to EB 1089. For a 70% reduction in clonogenicity, the radiation dose is 2-fold higher in the absence of EB 1089.

DISCUSSION

The present studies demonstrate that the hypocalcemic vitamin D_3 analog EB 1089 is more effective in inhibiting the growth of breast tumor cells which are VDR^+ (MCF-7) than isomolar concentrations of the parent compound, 1,25-$(OH)_2$-D_3. We failed to observe any direct evidence of cell killing in MCF-7 cells with either 1,25-$(OH)_2$-D_3 or EB 1089 with exposure times as long as 96 h, although it is possible that the reduction in the number of cells over time reflects both growth inhibition and (a limited degree of) cell death. While other laboratories have reported apoptosis in breast tumor cells (including MCF-7 cells) treated with vitamin D_3 compounds (*26–29*), the susceptibility to apoptosis appears to be limited to a relatively small fraction of the target cell population in these studies (i.e. between 5 and 15%) (*26, 27*). As a consequence of the prolonged survival of the bulk of the tumor cell population, most studies (both *in vitro* and *in vivo*) indicate that 1,25-$(OH)_2$-D_3,

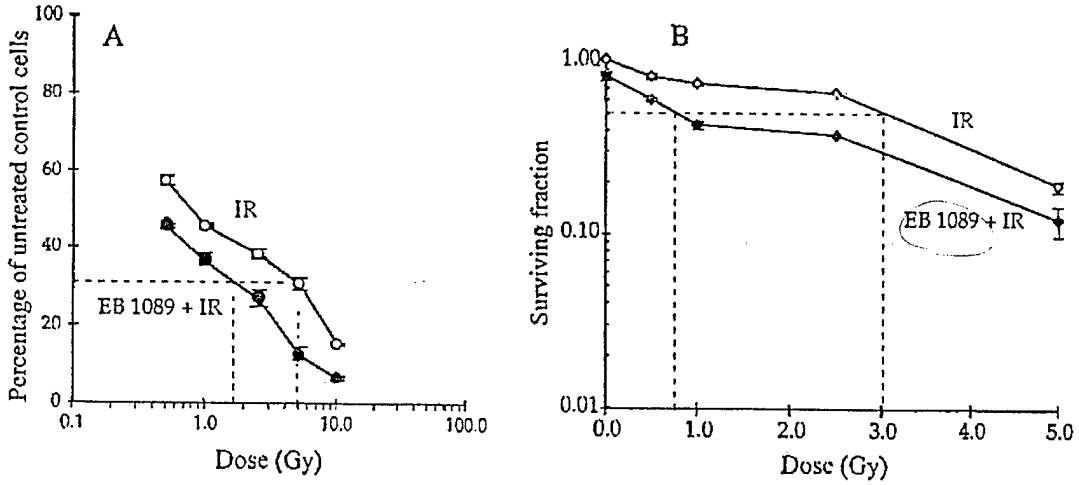

FIG. 6. Panel A: Response of MCF-7 cells to different doses of radiation (IR) with or without EB 1089 pretreatment. Cells were treated with EB 1089 (100 n*M*) for 48 h and replaced with fresh medium prior to irradiation (0.5–10 Gy). Cells were allowed to grow for an additional 96 h. Similar to the data shown in Fig. 1 about 50% growth inhibition was observed with 48 h of EB 1089 treatment. Results are expressed as percentages of untreated controls. Data represent means ± SEM of three experiments. Panel B: Clonal growth assay of MCF-7 cells after different doses of radiation (0.5–5 Gy) with or without EB 1089 pretreatment. Data represent means ± SEM of two independent experiments.

EB 1089 and KH 1060 are effective primarily in preventing tumor cell proliferation (*28–34*). Similarly, studies indicate that the primary response to radiation in both TP53 wild-type (MCF-7) and TP53-mutated breast tumor cells (MDA-MB 231) is a prolonged growth arrest (*4, 14*), although it is well established that many irradiated tumor cells ultimately die through reproductive cell death.

Treatment with agents that induce differentiation combined with conventional chemotherapy and radiotherapy has been shown to be effective in causing apoptosis in tumors of hematopoietic origin (*10, 13*). Additionally, a review of the literature suggests that the effectiveness of radiation can be enhanced in cells of human solid tumors with differentiating agents such as hexamethylene bisacetamide (HMBA) and *N*-methylformamide (NMF) (*35, 36*). Vitamin D_3 and its analogs, which are relatively nontoxic compounds, have been shown to cause differentiation of leukemia, lymphoma and breast tumor cells as well (*15–17*). Consequently, to circumvent the functional resistance to apoptosis observed in breast tumor cells, we combined 1,25-$(OH)_2$-D_3 and EB 1089 with radiation and observed a marked reduction in cell number. Similar to the results of other studies reported in the literature, our study comparing different human breast tumor cell lines substantiates the importance of the VDR for enhanced efficacy of the vitamin D_3 compounds (*23, 37, 38*). Nevertheless, the presence of VDR alone is not sufficient for sensitizing breast tumor cells to radiation since the combination was not effective in T-47D cells, which are VDR^+ but have mutated TP53. These studies suggest that the combination of EB 1089 and radiation is most effective in breast tumor cells that are both VDR^+ and TP53 wild-type. However, as these studies involved only one VDR^+ breast tumor cell line which is TP53 mutated, this conclusion requires further substantiation in isogenic VDR^+ cell lines which are TP53 wild-type or mutated.

Exposure to 1,25-$(OH)_2$-D_3 or EB 1089 prior to irradiation is not only effective in reducing final cell number significantly, but also appears to overcome the refractoriness of MCF-7 cells to radiation-induced apoptosis. The effectiveness of the combination of 1,25-$(OH)_2$-D_3 or EB 1089 with radiation is not due to an increase in radiation-induced initial DNA damage. To our knowledge, this is the first study showing the promotion of apoptosis in breast tumor cells by combining (in sequence) a differentiating agent with a classic DNA-damaging agent. However, studies by Light *et al.* (*18*) and Cho *et al.* (*39*) demonstrate a similar potentiating effect in inhibiting the growth of MCF-7 cells and murine squamous carcinoma cells by combining 1,25-$(OH)_2$-D_3 with platinum drugs. Furthermore, a recent report has demonstrated that 1,25-$(OH)_2$-D_3 enhances the response to adriamycin in the breast tumor cells, although this study did not address the issue of apoptosis (*40*).

If MCF-7 cells exposed to 1,25-$(OH)_2$-D_3 or EB 1089 with radiation are undergoing apoptosis according to our observation, there are a number of possible explanations for these findings. The promotion of apoptosis could be mediated through a reduction in levels of the anti-apoptosis proteins such as BCL2 in the face of an increase in TP53 (*12*), and possibly an increase in levels of the pro-apoptosis proteins such as BAX (*41, 42*); through abrogation of the TP53 and CDKN1A (formerly known as $p21^{waf1/cip1}$)-dependent G_1-phase arrest pathway in favor of TP53-dependent apoptosis (*43*); through prevention of a reduction in MYC or E2F1 protein levels, allowing for TP53 + MYC- or TP53 + E2F1-dependent apoptosis (*44–46*); or through suppression of the anti-apoptosis pathway induced by NFKB (*47*). Studies currently in progress are designed to discriminate between these different modes of action.

The role of cell cycle arrest, apoptosis and reproductive cell death in the sensitivity of tumor cells of nonhematological origin to anticancer drugs or radiation has been addressed by Waldman *et al.* (*48*) as well as in a recent review by Brown and Wouters (*49*). According to Waldman *et al.* although $CDKN1A^{-/-}$ cells undergo virtually complete apoptosis after irradiation, clonogenic surviving fractions after 10 Gy are identical to $CDKN1A^{+/+}$ cells which fail to undergo apoptosis. However, the cure rates (*in vivo*) for tumors derived from cells with normal and deficient CDKN1A cell cycle checkpoints were 0 and 18%, respectively, for a dose of 7.5 Gy and 0 and 38%, respectively for a dose of 15 Gy. These studies therefore suggest that apoptosis is critical for radiosensitivity *in vivo*. In contrast Brown and Wouters recently reviewed much of the literature relating to TP53 and the occurrence of apoptosis reaching the conclusion that TP53 status (and hence the absence or presence of apoptosis) did not necessarily predict overall sensitivity of nonhematological tumor cells to genotoxic agents.

In our own work, the presence of EB 1089 reduces the dose of radiation required for a specific effect on viable cell number by more than 60%. For instance, for a 70% reduction, a dose of 5 Gy is required with radiation alone, while a more clinically relevant dose of approximately 2 Gy is effective to the same extent in the presence of EB 1089. Similarly, our clonogenic survival assay indicates that the radiation dose required for a 50% reduction in clonogenic survival is approximately fourfold higher in the absence of EB 1089. At doses of radiation which alone produce more pronounced losses of clonogenicity, EB 1089 is somewhat less effective, but nevertheless produces a twofold enhancement of radiosensitivity. It is worthy of note that the results of these two assays tend to track quite closely. This may be due to the fact that the results in Fig. 6A were obtained 96 h after irradiation, an approach which does not have the same limitations as the short-term XTT assay described by Brown and Wouters (*49*). In fact, we have previously observed that the clonogenic assay for radiosensitivity closely parallels the trypan blue exclusion assay (unpublished results).

The shift in the dose–response curves in Figs. 6A and B appears to be related to the growth-inhibitory effects of

EB 1089 coupled with the promotion of apoptosis which now occurs in response to irradiation. Therefore, these data suggest that the potentiation of the effectiveness of radiation by EB 1089 in breast tumor cells may occur at least in part through the promotion of apoptosis in otherwise apoptosis-resistant cells. Whether apoptosis is necessary for the enhanced effectiveness of the combination is not certain, since loss of reproductive capacity could occur through a number of mechanisms other than or in addition to apoptosis, such as necrosis and reproductive cell death. Nevertheless, the ramification of this work is that it might be possible to use nontoxic analogs of 1,25-$(OH)_2$-D_3 to enhance the effectiveness of radiotherapy in the clinical treatment of breast cancer.

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Sustained enhancement of liposome-mediated gene delivery and gene expression in human breast tumour cells by ionizing radiation

P. T. JAIN* and D. A. GEWIRTZ

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Abstract.
Purpose: To investigate whether irradiation improves the delivery and expression of liposome–DNA complexes in human breast tumour cells.
Materials and methods: MDA-MB231 and MCF-7 human breast tumour cells were transfected with a liposomal SV40–luciferase complex and irradiated immediately after, at 24 h after or 24 h prior to transfection and in the presence or absence of serum. The amount of luciferase plasmid in the cell was evaluated after extraction by the Hirt procedure, while luciferase expression was measured using a luminescence assay.
Results: Ionizing radiation enhanced the liposome-mediated delivery and expression of the SV40–luciferase transgene in MDA-MB231 breast tumour cells both in the absence and presence of serum as well as in MCF-7 breast tumour cells. Improved transgene delivery and expression was observed at a clinically relevant dose of 2 Gy, and was dose-dependent over a dose range of 2–10 Gy. The effects of irradiation on transgene expression were observed with irradiation immediately prior to exposure of the cells to the liposome–transgene complex, with irradiation up to 24 h before or up to 24 h after initiation of exposure.
Conclusions: Irradiation at 24 h prior to exposure of breast tumour cells to the liposome–transgene complex appears to be the optimal approach for enhancing transgene delivery and expression. These findings suggest that ionizing radiation could promote the utility of gene therapy in the treatment of breast cancer.

1. Introduction

Ionizing radiation is used in conjunction with surgery and/or chemotherapy in the treatment of breast cancer and widely in the treatment of other solid malignancies. Previous studies from this laboratory (Watson *et al.* 1997) have indicated that breast tumour cells fail to undergo apoptotic cell death after irradiation. Therefore, the authors have been interested in identifying approaches for the delivery of apoptosis-inducing genes (such as *p53*) to breast tumour cells with the goal of promoting cell killing.

*Author for correspondence. Department of Medicine, Medical College of Virginia, Virginia Commonwealth University, PO Box 980230, Richmond, VA 23298, USA. e-mail: pjain@gems.vcu.edu
Departments of Pharmacology, Toxicology and Medicine, Medical College of Virginia, Virginia Commonwealth University, Richmond, VA, USA.

Recent studies have focused on the utilization of gene therapy to increase the capacity of ionizing radiation to achieve control of local tumours (Nguyen *et al.* 1997, Xu *et al.* 1997). These approaches have involved strategies such as replacement of the tumour suppresser gene, *p53*, and the expression of select cytokine genes including TNF-α and IFN-α for enhancement of radiosensitivity (Seung *et al.* 1995, Syljausen *et al.* 1997).

An additional strategy that has been identified in recent work is the utilisation of ionizing radiation to sensitize the target cell to incorporate exogenous genes through genetic recombination events (Stevens *et al.* 1996, Zeng *et al.* 1997). The present study demonstrates an alternative and complementary possibility, that ionizing radiation is capable of markedly increasing the cellular uptake and expression of an exogenous transgene in breast tumour cells. Unexpectedly, the influence of irradiation is evident over intervals as long as 24 h prior to or subsequent to exposure of cells to the liposome–DNA complex. Taken together with recent studies indicating that ionizing radiation can improve the efficiency of gene integration in mammalian cells through double-strand break rejoining and recombination (Zeng *et al.* 1997, Stevens *et al.* 1996), these findings suggest a novel rationale for the combination of ionizing radiation with gene therapy which may be useful in the clinical treatment of breast cancer as well as other solid malignancies.

2. Materials and methods

2.1. *Materials*

Dulbecco's modified Eagle medium (DMEM, 56-439) was obtained from Hazelton Research Products (Denver, PA, USA); L-glutamine, penicillin (10 000 U/ml), streptomycin (10 mg/ml) and foetal bovine serum were obtained from Whittaker Bioproducts (Walkersville, MD, USA); defined bovine calf serum was obtained from Hyclone Laboratories (Logan, UT, USA). Trypsin-EDTA, lipofectamine

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and optiMEM were obtained from GIBCO BRL (Gaithersburg, MD, USA). The pSV40–luciferase (Promega) was kindly provided by Dr Phil Hylemon and p-CMV-galactosidase (Clontech) was provided by Dr Eric Westin, both at the Medical College of Virginia, Richmond, VA, USA.

2.2. *Cell culture*

The MCF-7 breast tumour cell line was kindly provided by the laboratory of Dr Kenneth Cowan at the National Cancer Institute, Bethesda, MD, USA, while MDA-MB231 cells were provided by Dr Eric Westin at the Medical College of Virginia, Richmond, VA, USA. Cells were maintained in Dulbecco's minimal essential media (Hazelton Research Products) supplemented with 5% foetal calf serum (Life Technologies, Grand Island, NY, USA), 5% defined bovine serum (Hyclone Laboratories), glutamine (29.2 mg/100 ml), and penicillin/streptomycin (0.5 ml/100 ml) (Whittaker Bioproducts). Approximately $3–5 \times 10^4$ MCF-7 cells or 1×10^4 MDA-MB231 cells per well were subcultured in six-well plates and allowed to grow for 2–3 days to achieve 60% confluency prior to conducting the gene delivery experiments as described below.

2.3. *Preparation of the DNA–liposome complex and optimization of transfection*

The manufacturer's recommendations were followed for DNA–liposome complex preparation and optimization of the transfection condition to human breast cancer cells. Briefly, DNA was mixed with lipofectamine in serum-free optiMEM media and incubated at room temperature for 45 min with gentle shaking every 15 min. The optimal condition for transfection proved to be the use of 6 μl of lipofectamine. As uptake was saturated at 2–4 μg of DNA per ml, 2 μg of DNA (1:1 SV40–luciferase:CMV-β-galactosidase) and 6 μl of lipofectamine (Jain and Gewirtz 1997) were used throughout the study.

2.4. *Transgene–liposomes and radiation treatment*

Subconfluent cultures of MCF-7 or MDA-MB231 cells in six-well plates were washed with optiMEM and then exposed to 1 ml of media containing the DNA–liposome complex. After incubation for 5 h at 37°C, 1 ml of MEM plus 20% serum was added and cells were further incubated overnight at 37°C. Media was aspirated, the serum concentration was reduced to 10% and cells were maintained in MEM + 10% serum at 37°C for the indicated times. Cells were irradiated in an irradiator with a caesium-137 source emitting γ-radiation at the rate of 156.4 rad/min. The cells were exposed to varying doses of radiation (by varying the total time of exposure) either prior to or after initiation of the transfection procedure. The varying protocols of combining radiation with gene transfection are depicted in figure 1 and described in detail in §3. Except where otherwise indicated, irradiation was performed within 10 min after transfection.

2.5. *Luciferase reporter assay*

Cells transfected with pSV40–luciferase were washed twice with 2 ml PBS and lysed using 250 μl/well of reporter lysis buffer (Promega, Madison, WI, USA) containing 125 mM Tris, pH 7.8 with H_3PO_4, 10 mM EDTA, 10 mM DTT, 50% glycerol and 5% triton X-100, diluted 1:4 for 15 min at room temperature. The cell lysate was prepared by incubating the cells in a reporter cell lysate buffer for 10 min at room temperature and scraping cells using a rubber policeman. The cell lysate was collected in 1.5 ml microfuge tubes and centrifuged at 10 000 rpm for 2 min at 4°C. The supernatant was transferred to 1.5 ml Eppendorf tubes and stored at −70°C prior to the determination of luciferase activity.

The luciferase activity of the cellular extract was determined by mixing 20 μl of cell extract with 100 μl of Promega luciferase reagent containing 270 μM coenzyme A (lithium salt), 470 μM luciferin, 530 μM ATP, 20 mM tricine, 1.07 mM $(MgCO_3)$ $4Mg(OH)_2{*}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA and 33.3 mM DTT, pH 7.8 at room temperature. Relative light units (RLU) were measured for 20 s in a Berthold LB 9501 luminometer (Jain and Gewirtz 1998a).

In a parallel experiment, the transfected cells were trypsinized to quantitatively evaluate the viable cell number by trypan blue exclusion (Jain *et al.* 1992). The luciferase activity was expressed as RLU per viable cell.

2.6. *Extraction and quantitation of transfected DNA*

The luciferase plasmid DNA was extracted from MDA-MB231 breast cancer cells utilizing the standard Hirt protocol (Hirt 1967). Briefly, the transfected cells were suspended in 0.045 M Tris-borate, 0.001 M EDTA, 0.5% SDS and 1.6 M sodium chloride and digested overnight at 4°C. The cellular extract was pelleted and DNA was extracted twice with 200 μl of PCI (phenol:chloroform:isoamyl alcohol 25:24:1) and once with chloroform. The DNA was purified and precipitated using two volumes of ethanol, 0.02 M

sodium chloride, incubation at −80°C for 30 min and then pelleted at 14 000 rpm for 15 min at 4°C. The DNA was resuspended in an appropriate volume of sterile water in proportion to the viable cell number in order to maintain equivalent DNA concentrations in control and irradiated cells. DNA extracted from approximately 500 000 cells was loaded into each lane of an 0.8% agarose gel in TBE buffer containing ethidium bromide, and electrophoresis was performed at 90 mV for 3–4 h. Densitometric analysis was performed to quantitate the extracted DNA in each lane.

2.7. *Statistical analysis*

All experiments were repeated at least once. The radiation-treated cells were compared with controls by ANOVA; a p-value of <0.05 was considered to be statistically significant. The statistical analysis was performed utilizing Statview 512™ McIntosh statistical software.

3. Results

Initial studies were designed to determine the sequence(s) of irradiation and transfection with the liposome–DNA complex that would provide for optimal enhancement of gene uptake and/or expression. As shown in figure 1, the time at which cells are exposed to the liposome–transgene complex is considered to be the beginning of the cellular uptake phase (indicated as time 0). The time at which serum is added to terminate liposome-mediated uptake (5 h in the current experimental design) is considered to be the end of the uptake phase and the beginning of the 'post-uptake' phase. The times of irradiation during the pre-uptake or uptake/post-uptake phases are shown, respectively, as negative and positive numbers on the time scale.

In the experiment presented in figure 2, MDA-MB231 breast tumour cells were irradiated immediately after exposure to the complex of lipofectamine with the SV40–luciferase plasmid (i.e. with a delay of approximately 10 min between transfection and irradiation). Uptake (and expression) of the SV40–luciferase DNA (at 1 μg/ml) was increased approximately threefold by a radiation dose of 2 Gy (approximately 4% of the irradiated breast tumour cells were found to accumulate the transgene (Jain and Gewirtz 1998b)). As shown in figure 2, this increase is equivalent to the alteration in luciferase expression observed by doubling the amount of transfected plasmid.

In order to substantiate that ionizing radiation does in fact increase gene uptake into the cell, the transfected DNA was extracted from control and irradiated cells. Figure 3 indicates that the amount of DNA in the cells exposed to 2 Gy of ionizing radiation at the time of transfection (lane 4) was approximately threefold higher than that in cells that underwent the transfection protocol alone (lane 3). In duplicate experiments, the overall increase was 2.15 ± 0.8 fold. Both linear and supercoiled forms of the SV40 plasmid (lane 1) were used for the transfection procedure and both forms could be re-extracted from the cell lysate as shown in lane 2. Interestingly, the linear forms seem to be preferentially delivered

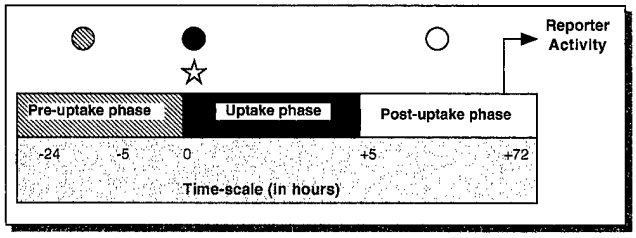

Figure 1. Schematic representation of the protocol for combined treatment of breast tumour cells with ionizing radiation (represented by circles) and the liposome–DNA complex (represented by a star). In the present study, 0 h refers to the time for initiation of the transfection procedure; all other times are indicated relative to this time point. Since the major proportion of transgene uptake occurs during the 5 h interval of exposure to the transgene–liposomal complex, this period is referred to as the uptake phase. The intervals prior to and post-exposure of the cells to the liposome–DNA complex are indicated as the pre-uptake and post-uptake phases, respectively. The luciferase reporter activity was evaluated 3 days after initiation of transfection as described in §2.

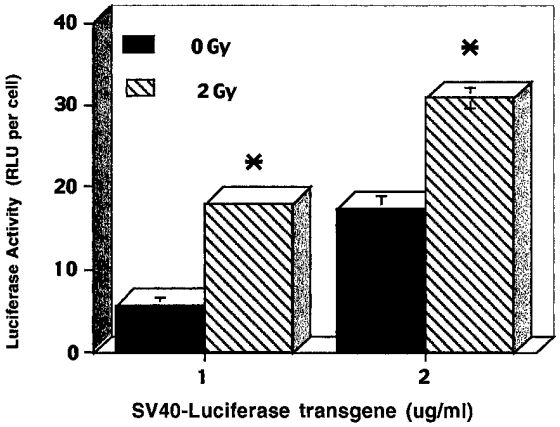

Figure 2. Influence of variations in the concentration of the SV40–luciferase gene on radiation- (2 Gy) induced improvement of liposomal transgene expression in MDA-MB231 human breast cancer cells. Each value represents the mean ± SEM of four replicate samples; * indicates that the values were significantly greater ($p<0.05$) than in unirradiated samples.

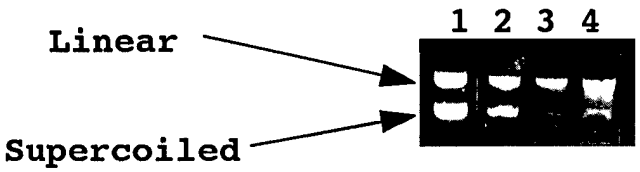

Figure 3. Extraction of SV40–luciferase DNA from irradiated and non-irradiated cells. The SV40–luciferase transgene was extracted from approximately 500 000 MDA-MB231 breast tumour cells by the Hirt method and electrophoresed on a 0.8% agarose gel. Lane 1: 1 μg of standard reference SV40–luciferase DNA; lane 2: SV40–luciferase that was added to an extract of untreated cells; lane 3: SV40–luciferase DNA extracted from unirradiated cells after transfection; lane 4: SV40–luciferase DNA extracted from cells after transfection and irradiation (2 Gy).

to the cells (lanes 3 and 4). These observations are in close agreement with the previous report of a radiation-induced increase in the number of stable transfectant colonies following delivery of the linear form of the lac-Z transgene into immortalized/transformed tumour cells (Steven *et al.* 1996).

A radiation dose of 2 Gy falls within the clinically relevant dose range used in radiotherapy (Rasmussen *et al.* 1997). In order to determine whether higher doses of ionizing radiation would produce a correspondingly greater increase in gene uptake and expression, the influence of 4 Gy and 10 Gy of ionizing radiation on SV40–luciferase expression were also evaluated. Figure 4A indicates that a radiation dose of 4 Gy increased transgene expression by eightfold while 10 Gy produced a 15-fold increase in gene expression in MDA-MB231 cells (three days after irradiation at doses of 2, 4 and 10 Gy, the number of viable MDA-MB231 cells was reduced by approximately 60%, 80% and 80%, respectively). The capacity of ionizing radiation to enhance gene expression was also evident in another breast tumour cell line. Figure 4B demonstrates a dose-dependent increase in transgene expression by ionizing radiation in MCF-7 breast tumour cells. At radiation doses of 2 Gy, 4 Gy and 10 Gy, expression was increased by 1.4, 3.9 and 8.0-fold, respectively. MCF-7 cells were less sensitive than MDA-MB231 cells in that the increase in expression was somewhat lower at comparable doses of radiation (three days after irradiation at doses of 2, 4 and 10 Gy, the number of viable MCF-7 cells was reduced by approximately 15%, 60% and 80%, respectively).

Since serum is known to interfere with transgene uptake (Hofland *et al.* 1996), it was important to determine whether the effects of ionizing radiation would be abrogated by the presence of serum. Figure 5 shows that, as expected, the efficiency of SV40–luciferase uptake by MDA-MB231 cells was significantly decreased in the presence of 5% serum (by approximately 80%). However, the capacity of a clinically relevant dose of ionizing radiation to enhance gene expression was similar in the presence and absence of serum. In this particular series of

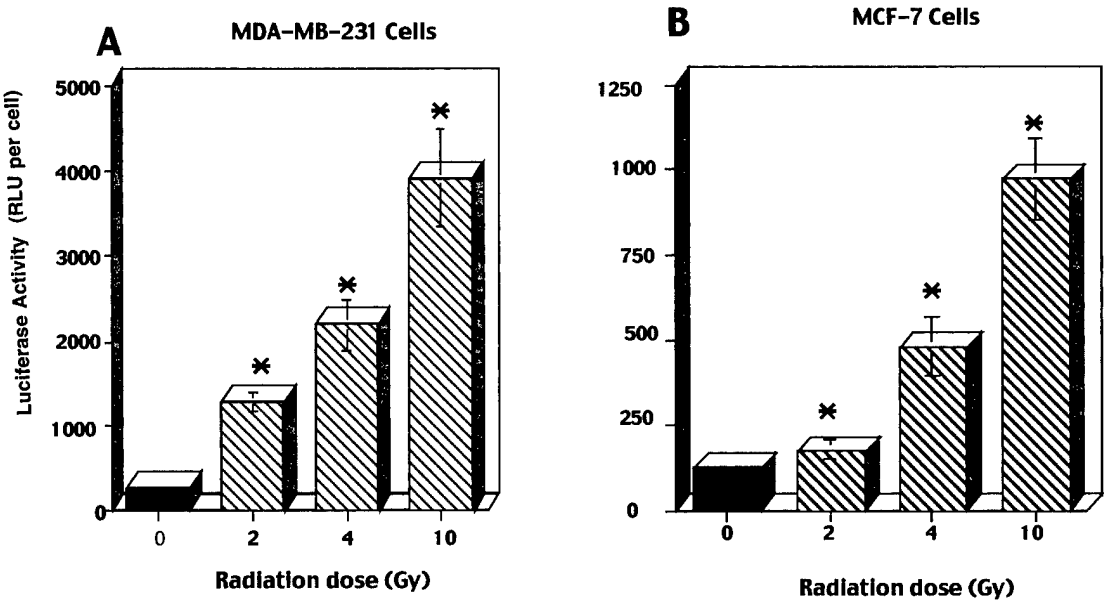

Figure 4. Dose-dependent increase in expression of the liposomal SV40–luciferase transgene by ionizing radiation in MDA-MB231 (A) and MCF-7 (B) human breast cancer cells. Cells were transfected with 2 μg DNA (SV40–luciferase:CMV-β-galactosidase 1:1) and irradiated with 0–10 Gy of ionizing radiation within 10 min after transfection. Reporter activity was determined 3 days post-transfection. Each value represents the mean ± SEM of four replicate samples; * indicates that the values were significantly greater ($p < 0.05$) than unirradiated samples.

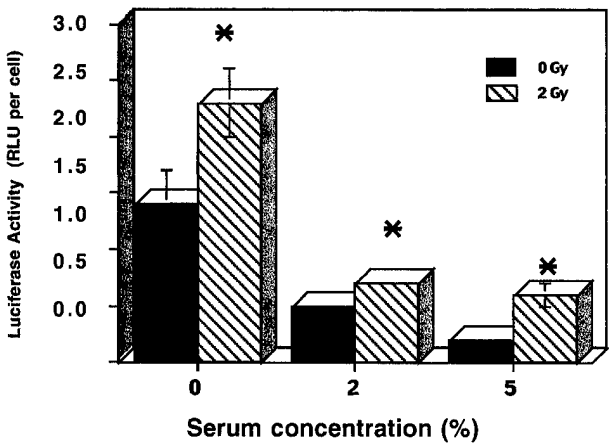

Figure 5. Influence of varying concentrations of serum on radiation-(2 Gy) induced improvement of liposome-mediated SV40–luciferase gene delivery and expression in MDA-MB231 human breast cancer cells. Each value represents the mean ± SEM of four replicate samples; * indicates that the values were significantly greater ($p < 0.05$) than unirradiated samples.

studies, 2 Gy of ionizing radiation increased gene expression by 1.7-fold in serum-free media and by 1.4- and threefold respectively in the presence of 2% and 5% serum.

In view of the possibility that ionizing radiation may ultimately prove useful in enhancing gene delivery and expression in clinical situations, it was important to determine the most effective sequence for irradiation and transfection. Irradiation of MDA-MB231 cells at various times within the 5 h uptake phase or the 6 h prior to the uptake phases was at least as effective as irradiation immediately after transfection (not shown). Consequently, studies were designed to assess the influence of irradiation on transgene expression when irradiation was performed

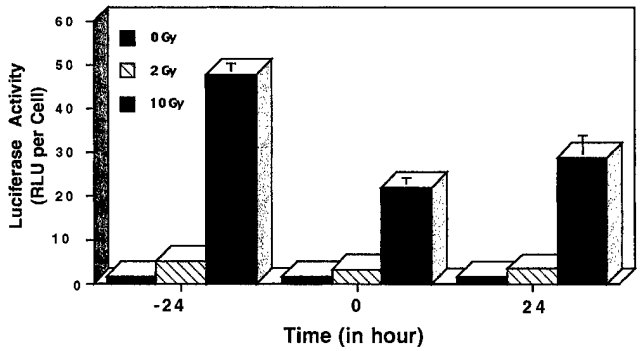

Figure 6. Influence of 2 Gy of ionizing radiation on the expression of liposomal SV40–luciferase in MDA-MB231 human breast cancer cells. The abscissa depicts the time in hours either prior to or post initiation of transfection. The uptake phase is considered to encompass the 5 h after initiation of transfection.

at longer intervals prior to uptake as well as during the post-uptake phase. Figure 6 indicates that irradiation 24 h prior to the uptake phase as well as 24 h post-uptake also resulted in enhancement of gene uptake and expression. Irradiation 24 h prior to exposure of the cells to the liposomal–transgene complex appeared to provide the greatest improvement in gene expression.

4. Discussion

Ionizing radiation is one of the primary modalities utilized in the treatment of localized breast cancer (Early Breast Cancer Trialists' Collaborative Group 1995). Radiation is thought to cause tumour regression primarily through the induction of apoptotic cell death consequent to DNA damage (Nelson and Kastan 1994). However, it has been previously reported that ionizing radiation induces growth arrest in the absence of apoptotic cell death in two breast tumour cell lines (Watson *et al.* 1997); furthermore, the authors have established that MCF-7 breast tumour cells are relatively refractory to DNA-damage-induced apoptosis (Fornari *et al.* 1996, Gewirtz *et al.* 1998). These findings imply that arrest of breast tumour cell growth by DNA damaging drugs or by ionizing radiation may permit the survival of a subpopulation of cells that could resume growth, leading to recurrence of the disease. Consequently, it appears that the clinical effectiveness of radiation in the treatment of breast cancer could be improved by promoting apoptotic cell death.

Recent studies have demonstrated that combining ionizing radiation with the *p53* transgene or a radiation-inducible transgene can re-establish cell death in otherwise radioresistant cells (Jain and Gewirtz 1997, Pirollo *et al.* 1997, Xu *et al.* 1997). As cell death appears to be inducible through the delivery and expression of cytotoxic and/or apoptotic transgenes, approaches for increasing the amount of transgene expressed in the cell nucleus should improve the effectiveness of gene delivery and expression systems.

This report indicates that ionizing radiation enhances the delivery and expression of a liposome–DNA complex. These effects of ionizing radiation are dependent on the dose of radiation and are evident even in the presence of serum. Radiation-induced enhancement of transgene uptake and expression is effective when cells are irradiated as long as 24 h before or after transfection with the liposome–DNA complex. These observations suggest that a relatively broad therapeutic window may be available for combining these two modalities in order to maximize the effectiveness of protocols involving the delivery of exogenous transgenes.

The mechanistic basis for the observed effects of irradiation on gene uptake and expression is currently under investigation. The authors are inclined to rule out the direct effects of DNA damage since this damage is known to be reversed within minutes (Bunch *et al.* 1995, Schwartz *et al.* 1995). Rather, the effects of irradiation in the experimental system could be related, at least in part, to alterations in the membrane and/or nuclear transport function (Strassle *et al.* 1991, Berroud *et al.* 1996) that may be associated with cells preparing to undergo growth arrest. Alternatively, radiation could produce sustained effects on specific gene functions, which directly or indirectly influence transport processes (Haimovitz-Friedman *et al.* 1994, Kasid *et al.* 1996). Another possibility is that alterations in intracellular calcium pools by irradiation (Floersheim 1993, Dreval 1994, Todd and Mikkelsen 1994) may influence DNA passage across cytoplasmic or nuclear membranes.

Since the expression of luciferase is under the control of a constitutive CMV promoter, the observed enhancement of activity is likely to be, at least in part, a consequence of the entry of additional copies of the transgene into the cell. This idea is supported by the extraction of increased levels of the transgene from cells after simultaneous irradiation and transfection. However, the extent of the increase in gene uptake may not be sufficient to account for the increase in gene expression. Alternatively, as entry of the transgene into the cell nucleus is required for gene expression (Dean 1997), radiation may be facilitating DNA transport from the cytoplasm to the nucleus. The fact that radiation 24 h post-transfection promotes enhanced gene expression even though the lipid–DNA complex is absent from the incubation medium, is consistent with the idea that radiation facilitates access of the transgene to the cell nucleus. In this context, it has been suggested that breakdown of the nuclear membrane during the transition between G2 and M could facilitate the entry of exogenous DNA (Nicolau and Sene 1982). Clearly there are other possible mechanisms to consider that could also be responsible for the observed increase in gene expression. These include, but are not limited to, increased stability of the vector, message, or protein product or even increased transcription of message.

Stevens *et al.* (1996) recently reported that DNA-damaging agents including ionizing radiation improve transfection efficiency; these studies were based on the integration of a transgene into the host genome, resulting in stably transfected colonies that were selected after 2–3 weeks. These investigators propose that as a consequence of DNA damage, alterations in gene expression in anticipation of DNA repair may influence the recombination process and thus enhance incorporation of the exogenous transgene. It is important to indicate how the findings presented in this report differ from (and complement) the work of this research group. The authors' studies utilize a luciferase transgene driven by a constitutive promoter that is transiently transfected into the target cells and does not require genomic integration for expression. During the (3–4 day) course of the experiment it is unlikely that significant numbers of stably transfected colonies would be formed. Although genomic integration cannot be completely ruled out in the studies where irradiation was performed simultaneously with or subsequent to irradiation, the effectiveness of irradiation prior to transfection in enhancing gene expression argues against DNA damage and subsequent gene integration during repair as contributory factors to the enhancement of gene expression by radiation.

Findings of a marked enhancement in transgene delivery to the cell and increased gene expression indicates that more transgene molecules should also be available in the nucleus for the recombinant events described by Stevens *et al.* (1996). Consequently, taken together with the work of these investigators (Stevens *et al.* 1996, Zeng *et al.* 1997) the current observations suggest that irradiation should be capable of increasing both initial gene transfer and expression, and of enhancing long-term stable transfection through genomic recombination and integration. These findings may prove relevant for the establishment of clinical protocols involving conventional radiation therapy combined with gene therapy.

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Estradiol enhances liposome-mediated uptake, preferential nuclear accumulation and functional expression of exogenous genes in MDA-MB231 breast tumor cells

Pramod T. Jain [a], Prem Seth [b], David A. Gewirtz [a,*]

[a] *Departments of Pharmacology, Toxicology and Medicine, Medical College of Virginia, Virginia Commonwealth University, P.O. Box 980230, Richmond, VA, USA*

[b] *National Cancer Institute, National Institute of Health, Bethesda, MD, USA*

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Abstract

Exposure of p53 mutated estrogen-receptor-negative MDA-MB231 human breast tumor cells to a pharmacological concentration of estradiol enhances liposome-mediated uptake and expression of SV-40 luciferase. Unexpectedly, the effect of estradiol on SV-40 expression is evident even when estradiol exposure occurs after the initial uptake phase; this suggests that estradiol may influence gene expression by mechanisms other than increasing gene uptake alone, such as altering the intracellular distribution of the gene. We determined that while uptake of SV-40 luciferase is increased only three-fold by estradiol, there is a 30-fold increase in the nuclear/cytoplasmic ratio of the gene. In order to demonstrate that the influence of estradiol on gene uptake and expression is translated into a functional response, the effects of estradiol on the function of an exogenous gene, in this case the apoptotic function of p53, were assessed in the p53 mutated MDA-MB231 breast tumor cell. While liposome-mediated delivery of CMV-p53 alone was ineffective in promoting cell death, incubation with estradiol and the liposomal p53 complex resulted in a two-fold increase in cell killing over that observed in cells transfected with the corresponding mock vector (empty vector for p53). Evidence that cell killing was occurring through apoptosis included apoptotic body formation, cell shrinkage and an increase in fluorescence after terminal transferase end-labeling. The capacity of estradiol to promote apoptosis in MDA-MB231 cells by a p53–liposome complex is likely to be related to the preferential redistribution of the gene from the cytoplasm to the nucleus which could occur during both the uptake and post-uptake phases. Consequently, although direct effects on gene expression, and the stability of message and protein cannot be ruled out, the predominant effect of estradiol in this experimental system appears to be to influence DNA translocation from the cytoplasm to the cell nucleus.

1. Introduction

Both viral and non-viral systems can be utilized for the delivery of exogenous genes into cells and are being developed for use in gene therapy [1]. The most widely used non-viral gene delivery system relies on the use of cationic lipids to form complexes with DNA [2], although non-viral delivery systems are generally relatively inefficient [3]. We have recently reported that the use of pharmacological concentrations of estradiol as well as ionizing radiation can enhance the efficiency of liposomal-mediated gene uptake and expression in the breast tumor cell

* Corresponding author. Department of Medicine, Medical College of Virginia, P.O. Box 980230, Richmond, VA 23298, USA. Fax: +1 804-828-8079; E-mail: ~~pjain~~gewirtz@hsc.vcu.edu

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[4,5]. The current studies were performed to determine how estradiol influences select components of this process including gene uptake, intracellular distribution between the cytoplasmic and nuclear compartments and gene function.

We previously demonstrated that the non-physiological concentration of estradiol used in these studies could increase gene uptake and expression in both estrogen-receptor-positive MCF-7 and estrogen-receptor-negative MDA-MB231 breast tumor cells. This finding is consistent with an earlier report indicating that the effects of pharmacological concentrations of estradiol are not dependent on the estrogen-receptor status of the breast tumor cell [7]. The current work is focused exclusively on MDA-MB231 cells as these cells have mutant p53 [8] and mutations in p53 have been shown to prevent apoptosis [9,10]. Since adenoviral delivery of p53 has been shown to promote apoptosis in p53 mutant MDA-MB-231 cells [11], we reasoned that these cells could be used to assess gene function if the delivery of p53 in the presence of estradiol were to promote apoptosis.

The present studies demonstrate that estradiol increases gene uptake, and produces a profound alteration in the intracellular distribution of an exogenous gene. Furthermore, the function of the exogenous gene remains intact as these effects of estradiol are accompanied by the induction of apoptosis when the exogenous gene delivered to the cell is p53.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM, 56–439) was obtained from Hazelton Research Products, Denver, PA; L-glutamine, penicillin (10 000 U/ml), streptomycin (10 mg/ml), and fetal bovine serum were obtained from Whittaker Bioproducts, Walkersville, MD; defined bovine calf serum was obtained from Hyclone Laboratories, Logan, Utah. Trypsin-EDTA, lipofectamine, and optiMEM were obtained from Gibco-BRL (Gaithersburg, MD). The pSV-40–luciferase (Promega) was kindly provided by Dr. Phil Hylemon. The p-CMV-p53 and p-CMV-mock p53 were constructed as previously described [9].

2.2. Cell culture

The MDA-MB-231 cells were obtained from Dr. Eric Westin at the Medical College of Virginia of Virginia Commonwealth University, Richmond, VA. Cells were maintained in Dulbecco's minimal essential medium (Hazelton Research Products, Denver, PA) supplemented with 5% fetal calf serum (Life Technologies, Grand Island, NY), 5% defined bovine serum (Hyclone Laboratories, Logan, UT) glutamine (29.2 mg/100 ml), amphotericin B (5 μg/ml) (Sigma), and penicillin/streptomycin (0.5 ml/100 ml) (Whittaker Bioproducts, Walkersville, MD). Approximately, 1×10^4 MDA-MB-231 cells per well were subcultured in six-well plates and allowed to grow for 2–3 days so as to achieve 60% confluency prior to conducting the gene delivery experiments described below.

2.3. Preparation of DNA–liposome complex and optimization of transfection conditions

The manufacturer's recommendations were followed for DNA–liposome complex preparation and optimization of the transfection conditions to human breast cancer cells. Briefly, DNA was mixed with lipofectamine in serum free optiMEM media and incubated at room temperature for 45 min with gentle shaking every 15 min. Optimal conditions for transfection proved to be the use of 6 μl of lipofectamine. As 2–4 μg of DNA per ml gave adequate results, 2 μg of DNA (1:1::SV-40 luciferase: CMV-β-galactosidase) and 6 μl of lipofectamine [4,5,12] were used throughout the study.

2.4. Transfection procedure and estradiol co-treatment

Estradiol was dissolved in a mixture of ethanol/polyethylene glycol (at a ratio of 45:55), a vehicle non-toxic to human breast cancer cells [13]. MDA-MB-231 cells in six well plates were washed with optiMEM, exposed to the DNA–liposome complex in the presence of either vehicle or various concentrations of estradiol (in a volume of 1 ml) and incubated at 37°C for 5 h. An additional 1 ml of MEM

media containing 20% serum and either vehicle or estradiol was added and the incubation continued overnight at 37°C. The media was decanted and replaced with MEM containing 10% serum and cells were incubated at 37°C for the indicated times. In selected experiments, the cells were exposed to estradiol either during the uptake or post-uptake phases, as well as during both uptake and post-uptake phases, as shown in Fig. 1.

2.5. Luciferase reporter assay

Cells transfected with DNA were washed twice with 2 ml PBS, and lysed using 250 μl/well of reporter lysis buffer (Promega, Madison, WI) containing 125 mM Tris, pH 7.8 with H_3PO_4, 10 mM EDTA, 10 mM DTT, 50% glycerol and 5% triton X-100, diluted 1:4 for 15 min at room temperature. The cell lysate was scraped using a rubber policeman, collected in 1.5 ml microfuge tubes and centrifuged at 10 000 rpm for 2 min at 4°C. The supernatant was transferred to a 1.5-ml Eppendorf tube and stored at −70°C prior to the determination of luciferase activity.

The luciferase activity of the cellular extract was determined by mixing 20 μl of cell extract with 100 μl of Promega Luciferase reagent containing 270 μM coenzyme A (lithium salt), 470 μM luciferin, 530 μM ATP, 20 mM tricine, 1.07 mM $(MgCO_3)$ $4Mg(OH)_2{\cdot}5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM EDTA and 33.3 mM DTT, pH 7.8 at room temperature. Relative light units (RLU) were measured for 20 s in a Berthold LB 9501 luminometer [4,5,10].

In parallel experiments, the transfected cells were trypsinized to quantitatively evaluate viable cell number by trypan blue exclusion [14]. The luciferase activity was expressed as RLU per viable cell.

2.6. Cell fractionation

For the determination of intracellular distribution of the transfected gene, the transfected cells were pelleted and processed as described below. The cells were labeled with [^{3}H]thymidine at 37°C for 24 h. The radioactive media was decanted and the cells were pelleted and suspended in 1 ml triton based buffer (10 mM Tris-HCl, 10 mM KCl, 1.5 mM $MgCl_2$, 0.25% triton). Cells were homogenized by repeated passage (×5) through a 26.5-gauge needle attached to a 1-ml disposable syringe. The solution was centrifuged at 10 000 rpm for 10 min. The supernatant (cytoplasmic fraction) was removed and the nuclear pellet was resuspended in sucrose based buffer (0.25 M sucrose, 10 mM NaCl, 10 mM Tris HCl, 1.5 mM $MgCl_2$) together with 150 μl of detergent (10% sodium deoxycholate and 10% Tween-40) and vortexed for 30 s to lyse nuclei. The purity of nuclear and cytoplasmic fractions were verified, respectively, based on radioactive thymidine incorporation by tricholoroacetic acid precipitation and colorimetric assessment of lactate dehydrogense enzyme activity.

2.7. Extraction and quantitation of transfected DNA

The luciferase plasmid DNA was extracted from MDA-MB231 breast cancer cells utilizing the standard Hirt protocol [15] which extracts low molecular weight DNA through the preferential precipitation of cellular DNA in the presence of SDS and NaCl. Briefly, the transfected cells were suspended in 0.045 M Tris-borate, 0.001 M EDTA, 0.5% SDS and 1.6 M sodium chloride and digested overnight at 4°C. The cellular extract was pelleted and DNA was extracted twice with 200 μl of PCI (phenol/chloroform/isoamyl alcohol, 25:24:1) and once with chloroform. The DNA was purified and precipitated using two volume of ethanol, 0.02 M sodium chloride, incubated at −80°C for 30 min and then pelleted at 140000 rpm for 15 min at 4°C. The DNA was resuspended in an appropriate volume of sterile water in proportion to the viable cell number so as to maintain equivalent DNA concentrations in control and irradiated cells. DNA extracted from approximately 500 000 cells was loaded into each lane of an 0.8% agarose gel in TBE buffer containing ethidium bromide, and electrophoresis was performed at 90 mV for 3–4 h. Densitometric analysis was performed to quantitate the extracted DNA in each lane.

2.8. Statistical analysis

All experiments were repeated at least once. The estradiol-treated cells were compared with controls by ANOVA; a P-value of <0.05 was considered to be statistically significant. The statistical analysis

was performed utilizing Statview 512TM McIntosh statistical software.

3. Results

3.1. *Influence of estradiol exposure during liposome-mediated gene uptake and post-uptake phases on gene expression*

We have previously reported that exposure of either estrogen-receptor positive MCF-7 or estrogen-receptor negative MDA-MB231 breast tumor cells to a pharmacological concentration of estradiol increases the expression of luciferase from an SV-40–luciferase reporter gene transfected into the cells by a liposome-mediated transfection protocol [4]. These studies were performed as shown by arrow C in Fig. 1, with continuous exposure of the cell to estradiol during both the uptake phase (when cells are exposed to the liposomal DNA complex) as well as during the post-uptake phase (after the liposomal DNA complex has been withdrawn from the incubation medium).

Estradiol may influence subcellular processes in cytoplasmic and nuclear compartments[7,16] as well as cellular membrane stability [17]. Thus, it is possible that the observed effects of estradiol represent alterations in the intracellular distribution of the exogenous gene as well as enhanced gene uptake across the cell membrane. If this is the case, then estradiol should be capable of influencing gene expression even during the post-uptake phase, when effects on uptake across the membrane are likely to be negligible.

In order to determine whether the influence of estradiol on gene expression could be related to translocation and expression independent of gene uptake, we evaluated the effects of estradiol on gene expression with estradiol exposure during the post-uptake phase (i.e. after removal of the liposomal complex as shown by arrow B in Fig. 1). We further compared the relative effects on gene expression of estradiol exposure during both the uptake and post-uptake phases (arrow C in Fig. 1) with estradiol exposure during either phase (arrows A and B) alone.

Fig. 2 shows that, as expected, luciferase expression from the SV-40–luciferase reporter gene was increased by exposure to estradiol during the uptake phase. In addition, a significant increase in luciferase expression was also observed upon exposure to estradiol during the post-uptake phase. As would be expected from these and previous observations [4], the increase in expression following exposure to estradiol during both the uptake and post-uptake phases was greater than that with estradiol treatment during the uptake or post-uptake phases alone.

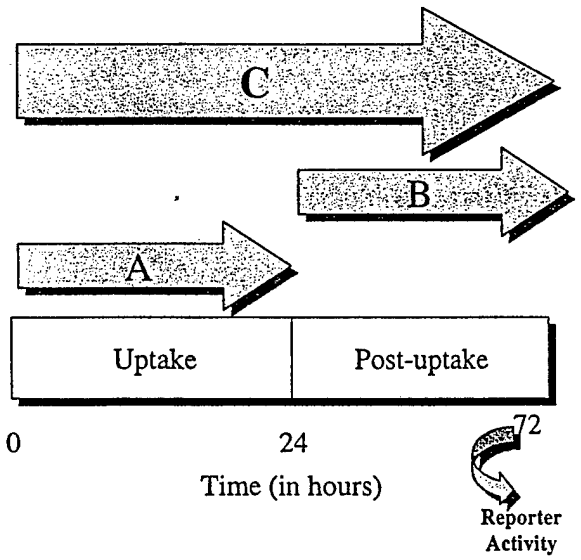

Fig. 1. Schematic representation of the protocol for liposomal gene delivery combined with estradiol in MDA-MB-231 human breast cancer cells. Phases of gene-delivery: transfection with the liposomal SV-40–luciferase gene was initiated at time 0 and continued at 37°C for 24 h (uptake phase). After the gene formulation was removed and replaced by fresh medium, incubation was continued for an additional 48 h (post-uptake phase). After 72 h the cell extract was prepared to evaluate reporter activity. Cells were exposed to estradiol during the uptake phase alone (A), the post-uptake phase alone (B) or during both the uptake and post-uptake phases (C).

3.2. *Influence of estradiol on intracellular gene distribution*

The observation that estradiol exposure even during the post-uptake phase increases gene expression supports the hypothesis that estradiol is having effects other than or in addition to enhancing gene delivery across the cell membrane. In order to determine how estradiol influences levels of the exogenous gene within the cell as well as its intracellular distribution, cells were transfected with the SV-40–lucifer-

ase–liposomal complex either with or without a 24-h co-exposure to estradiol. SV-40 luciferase was isolated from whole cell extracts as well as the nuclear and cytoplasmic fractions. The relative purity of the cytoplasmic and nuclear fractions was substantiated by demonstrating that greater than 95% of cellular lactate dehydrogenase activity was associated with the cytoplasmic extract and similarly that greater than 95% of [^{3}H]thymidine incorporation was evident in the nuclear extract (Fig. 3).

In the representative experiment assessing total gene uptake into the cell presented in Fig. 4A, cells treated with estradiol during the uptake phase are shown to accumulate significantly higher levels of the SV-40–luciferase transgene than the corresponding controls transfected with SV-40 luciferase in the absence of estradiol. Fig. 4B indicates that there was a 2.8 ± 0.9-fold increase in the delivery of the luciferase gene by estradiol.

Although there is a significant increase in gene uptake, this is clearly insufficient to explain 30-fold increase in gene expression which occurs as a result of estradiol exposure during the uptake phase. In the studies presented in Fig. 5A to establish whether estradiol affects the nuclear to cytoplasmic distribution

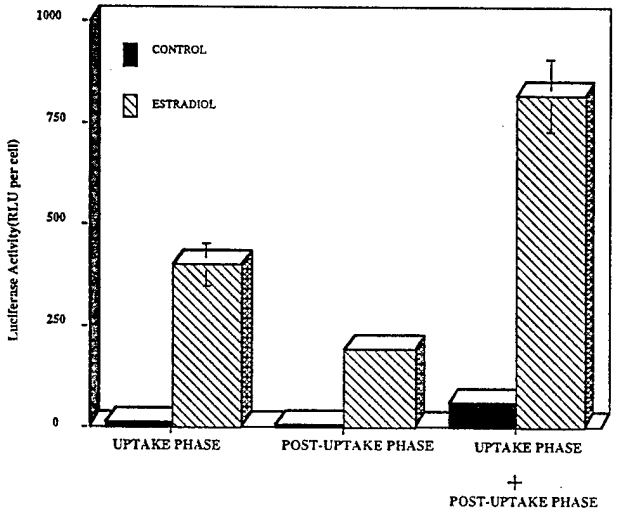

Fig. 2. Improvement of SV-40–luciferase gene expression in MDA-MB-231 human breast cancer cells following exposure to 100 μM estradiol during the uptake, post-uptake, and uptake/post-uptake phases. Note that maximal improvement in luciferase expression occurs following exposure to estradiol during both the uptake and post-uptake phases.

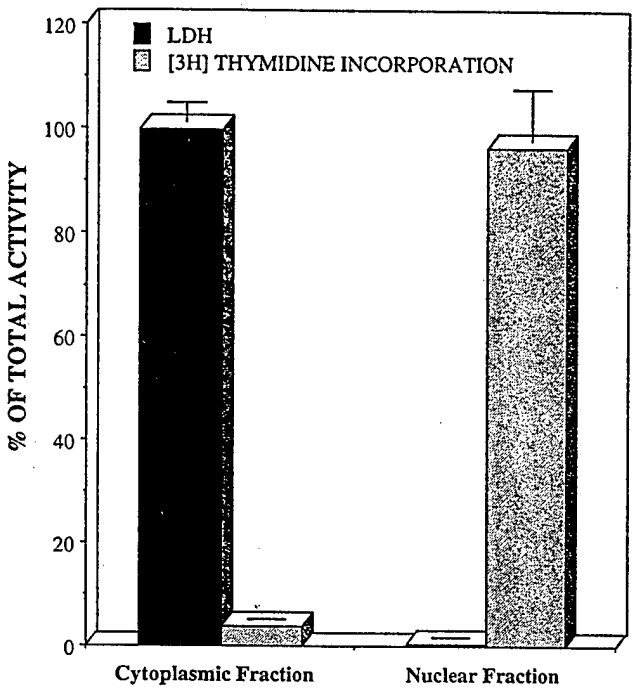

Fig. 3. Cytoplasmic and nuclear fractionation of cells. Lactate dehydrogenase and [^{3}H]thymidine incorporation were used as cytoplasmic and nuclear markers for assessing the purity of the separated fractions from which the exogenously transfected DNA was extracted (as shown in Fig. 5).

of the exogenous transgene, it is evident that estradiol essentially reversed this distribution ratio. That is, a far greater amount of the luciferase gene accumulated into the nucleus of estradiol-treated cells as compared to those treated with the vehicle control. Correspondingly, a marked decrease was evident in cytoplasmic SV-40–luciferase in the estradiol-treated cells. The alteration in intracellular distribution of the luciferase gene from the cytoplasm into the nucleus was quite pronounced. Fig. 5B indicates that there was a 28.8 ± 0.9 fold increase in the nuclear/cytoplasmic ratio of the SV-40–luciferase which approximates the increase in gene expression shown in Fig. 2.

3.3. *Promotion of apoptotic cell death in MDA-MB231 breast tumor cells by the combination of estradiol and the liposomal p53 complex*

The studies described above demonstrate increased gene uptake, redistribution of the gene from the cy-

toplasm to the nucleus and enhanced gene expression by estradiol. However, it is necessary to demonstrate that these effects of estradiol also serve to re-establish the functional response of the gene and that this function is pharmacologically significant. For this purpose, we chose to assess the capacity of estradiol to re-establish the apoptotic function of p53, as MDA-MB231 cells have a mutant p53 gene [8]. We reasoned that restoration of p53 to these cells might be sufficient to promote apoptotic cell death as has been shown in other studies where wild-type p53 was restored to p53 mutant cells [11]. Cells were transfected with either p53 under the control of a constitutive CMV promoter or with mock p53 (the plasmid originally used for cloning the p53 construct) using the standard Lipofectamine transfection protocol, but without estradiol treatment. Table 1 indicates

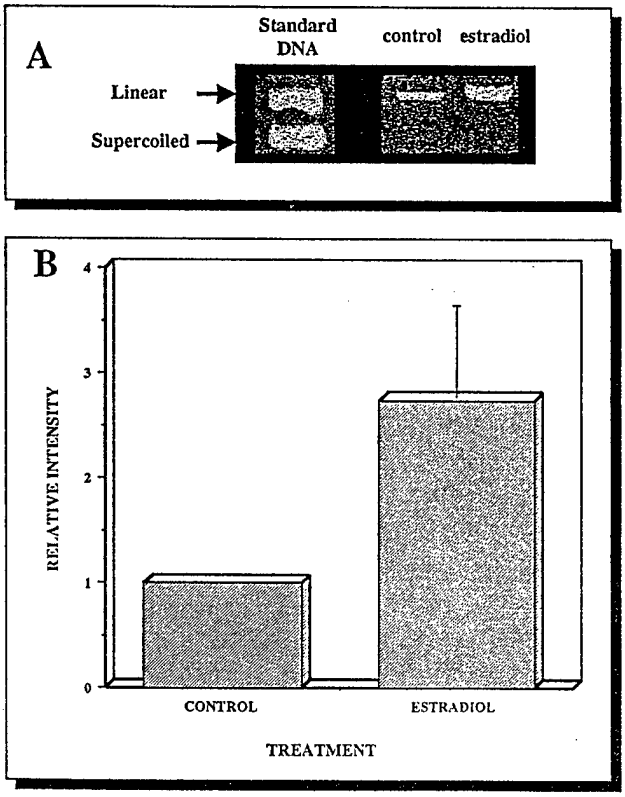

Fig. 4. Estradiol improves delivery of the SV-40–luciferase gene in MDA-MB-231 human breast cancer cells. Cells were exposed to 100 μM estradiol and the liposomal SV-40–luciferase complex for 24 h. (A) SV-40–luciferase plasmids isolated from the entire cells and separated by agarose gel electrophoresis. (B) The extent of improvement in luciferase gene delivery from three different experiments ± S.E.M.

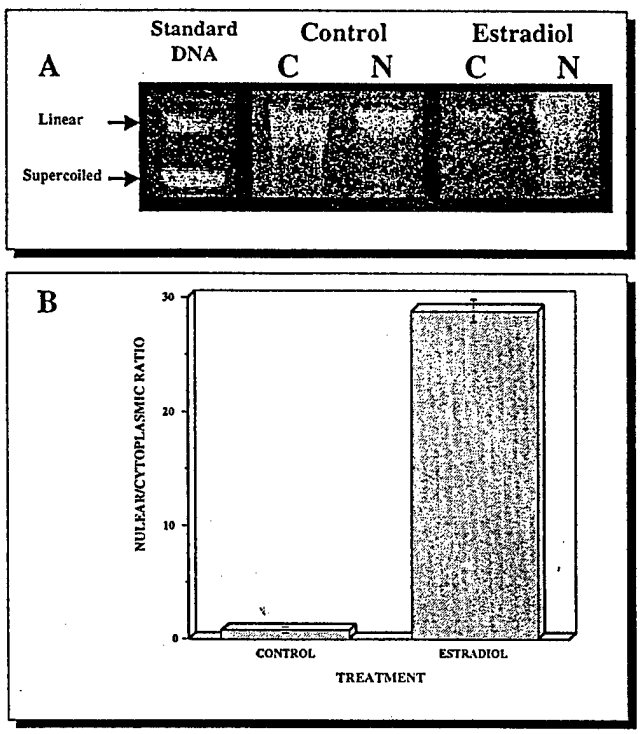

Fig. 5. Estradiol alters the intracellular distribution of the SV-40–luciferase gene in MDA-MB-231 human breast cancer cells. Cells were exposed to 100 μM estradiol and the liposomal SV-40–luciferase complex for 24 h. (A) SV-40–luciferase plasmids extracted from cytoplasmic and nuclear fractions. (B) The nuclear/cytoplasmic ratio for the luciferase gene in two separate experiments ± S.E.M.

that there was no difference in the extent of cell death (30–35% of the cell population) in the absence or presence of p53 under these experimental conditions (i.e. transfection in the absence of estradiol). We concluded that the observed toxicity was related to the transfection protocol and that p53 was not influencing cell viability. The lack of a functional p53 response was likely to be due to insufficient levels of the gene within the cell or limited distribution of the gene to the nucleus.

Consequently, cells transfected with the liposome–p53 were incubated with estradiol during both the uptake and post-uptake phases in order to both increase levels of the gene within the cell and to enhance its translocation to the nucleus. Under these conditions, cells transfected with p53 demonstrated a marked enhancement of cell killing. Table 1 indicates that incubation with estradiol essentially doubled the number of non-viable cells as compared to transfection with p53 in the absence of estradiol. Although

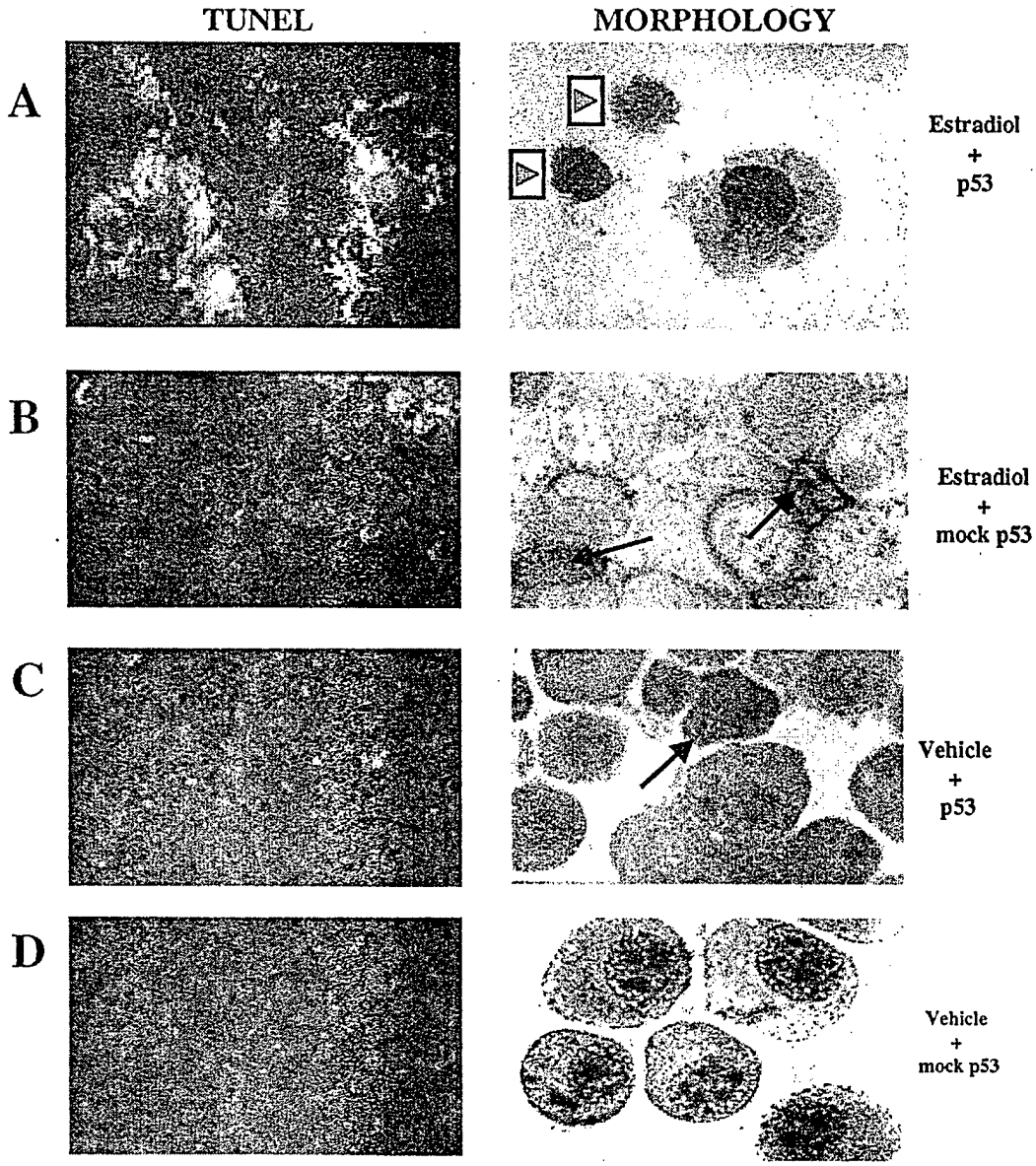

Fig. 6. Fluorescent staining (TUNEL assay) and morphological evaluation (phase contrast microscopy) of apoptotic cell death in MDA-MB-231 human breast cancer. Cells were treated with the liposomal CMV-p53 gene complex and estradiol (100 μM) during both the uptake and post-uptake phases. For the TUNEL assay, cells were incubated with fluorescent labeled antibodies. For morphological evaluation, cells were incubated with Giemsa–Wright stain. Note formation of apoptotic bodies (arrowhead) and cell shrinkage (arrows) in the cells treated with both estradiol and the p53 gene (A). These typical characteristics of apoptosis were negligible in the cells treated with estradiol/mock p53 (B), vehicle/p53 (C) or vehicle/mock p53 (D).

exposure to estradiol during either the uptake or post-uptake phases alone significantly enhances reporter transgene expression (as shown in Fig. 2), neither of these approaches was sufficient for the induction of apoptosis (data not shown).

To ascertain that cell death which occurred after transfection with p53 in the presence of estradiol was indeed related to an apoptotic mechanism, we evaluated various morphological and biochemical indicators of apoptosis. As shown in Fig. 6, phase contrast microscopy clearly indicated the presence of shrunken cells and apoptotic nuclear bodies after treatment with estradiol and transfection with p53. These characteristics were negligible in the MDA-MB-231 cells treated with estradiol and a mock p53 vector (panel B), the vehicle and p53 (panel C), or with vehicle and

mock p53 vectors (panel D). Similarly, the TUNEL assay demonstrated an intense fluorescent signal in the MDA-MB-231 cells treated with estradiol and p53 (panel A), which was not evident for any of the other experimental condition.

4. Discussion

Previous studies in this laboratory have demonstrated that pharmacological concentrations of estradiol increase the expression of genes delivered by a liposome-mediated protocol in both estrogen-receptor-positive MCF-7 and estrogen-receptor-negative MDA-MB231 breast tumor cells [6]. These observations indicate that the effects of a supra-physiological concentration of estradiol on gene uptake and expression in the breast tumor cell is independent of estrogen-receptor status. In the current work, we have further established that the mechanism for improving transgene expression by estradiol includes its influence on gene uptake across the cell membrane as well as redistribution of the transgene from the cytoplasm into the nucleus. In fact, the most pronounced effect of estradiol appears to be the 30-fold increase in the nuclear to cytoplasmic ratio rather than the 2–3-fold increase in gene uptake. The capacity of estradiol to alter the nuclear to cytoplasmic distribution of the gene is further supported (albeit indirectly) by the observation that estradiol enhances gene expression during the post-uptake phase – when transport of the gene across the cell membrane is expected to be negligible.

A number of mechanisms could be responsible for the effects of estradiol on transmembrane gene transport, the redistribution of the transgene between the nuclear and cytoplasmic compartments and the enhancement of gene expression. These include effects on cell membrane stability [17], deregulation of ions such as calcium [16], alteration in the nuclear membrane [16] and of cyto/nuclear-skeletal proteins [7,18]. Estradiol could also influence the function of the exogenous gene at the transcriptional level by stimulating activity of the promoter, post-transcriptionally at the level of mRNA stability or at the translational and post-translational levels, respectively, by increasing protein synthesis or the stability of the expressed protein.

It is of interest that apoptotic cell death could not be re-established simply through liposomal-mediated delivery of the p53 transgene to the MDA-MB231 cells. This could be related to the fact that most of the exogenous DNA is localized to the cytoplasmic fraction. However, when cells were exposed to estradiol during either the uptake or post-uptake phases alone, the liposomal p53 complex failed to promote apoptosis. This suggests that despite the effects of estradiol on the redistribution of the gene from the cytoplasm to the nucleus, there may still have been insufficient p53 in the cell to promote apoptosis. In contrast, when MDA-MB231 cells were exposed to 100 μM estradiol both immediately upon transfection with p53 as well as during the post-uptake phase, the cells apparently underwent apoptotic cell death. Thus, it appears that a threshold level of nuclear p53 gene expression may be required for the promotion of apoptotic cell death.

In summary, these studies indicate that exposure of the breast tumor cell to a pharmacological concentration of estradiol increases the liposome-mediated uptake, cytoplasmic to nuclear translocation, expression and function of an exogenous gene in the breast tumor cell. Therefore, estradiol has the capacity to alter fundamental aspects of cell function through estrogen-receptor-independent mechanisms.

Acknowledgements

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Free Radical-based DNA-cleaving Natural Products as Cancer Chemotherapeutic Agents

Lawrence F. Povirk

Department of Pharmacology and Toxicology

Medical College of Virginia, Virginia Commonwealth University

Richmond, VA 23298, U.S.A

1. Introduction

It is now more than 40 years since the discovery of phleomycin [1], the first of a large and still-growing family of radiomimetic free radical-based DNA-cleaving natural products and their synthetic analogues [2-4]. Despite the enormous efforts that have been devoted to chemical, biochemical, cellular, preclinical and clinical studies of these potent antibiotics, the phleomycin analogue bleomycin remains the only compound of this group that has found extensive use in cancer chemotherapy. For reasons that are not yet clear, the enediyne family of compounds (see Table I), have in general performed poorly in initial animal and clinical studies, and seem to lack the selective *in vivo* toxicity toward tumor cells that is characteristic of clinically useful DNA-damaging agents. Nevertheless, despite their apparent lack of intrinsic selectivity, the extraordinary cytotoxicity of some of these compounds, in the picomolar range and below, makes them particularly attractive as potential candidates for specific targeting to tumor cells by conjugation to tumor-specific ligands.

This review will attempt to summarize recent developments in the basic and preclinical study of the radiomimetic antibiotics, with emphasis on attempts to modulate and target their cytotoxicity.

2. Mechanisms of DNA cleavage

The enediynes all generate activated aromatic diradical forms through collapse of a highly strained enediyne ring [2-4]. In most cases this reaction is triggered by reaction with a sulfhydryl, as shown in Fig. 1 for calicheamicin. Recently, NMR-derived solution structures of DNA complexes with calicheamicin [16,17], esperamicin [18], and the nonprotein "chromophore" of neocarzinostatin (Zinostatin; NCS) [19] have confirmed previous inferences that the resulting diradical species bind in the minor groove in a stereospecific manner such that the radicals are strategically positioned to effect simultaneous hydrogen abstraction from deoxyribose moieties in both DNA strands, on a 2-base or 3-base 3′ stagger (Table I), resulting in double-strand breaks with 3′ overhangs. The abstraction in one of the strands is almost always from C-5′ of deoxyribose, resulting in a break with 3′-phosphate and 5′-aldehyde termini. In the complementary strand, hydrogen is abstracted either from C-4′, resulting in breaks with 3′-phosphoglycolate and 5′-phosphate termini; or from C-1′, resulting in labile C-1′-oxidized abasic sites that can decompose to form breaks with phosphates at both termini. Breaks resulting from C-1′ or C-4′ oxidations, but not those resulting from C-5′ oxidation, are accompanied by loss of a single DNA base at the break site. The enediynes differ substantially in the fraction of induced lesions that involve concomitant damage to both strands (Table I).

The bleomycins (Fig. 2), despite similarities to the enediynes in DNA damage and biological effects, have several distinguishing features. First, while the chemistry of enediyne activation is entirely organic, the free radical reactions of bleomycin are catalyzed by chelated iron [20-22]. Second, contrary to the 3′ stagger seen with enediynes, bleomycin induces damage either at the same sequence position in both strands or on a one-base 5′ stagger, resulting in double-strand breaks that either are blunt-ended or have single-base 5′ overhangs [23,24]. Third, contrary to the strictly stoichiometric action of the enediynes, bleomycin can undergo several catalytic cycles of oxidation-reduction and DNA damage before itself succumbing to irreversible free radical-mediated degradation [25].

Finally, since (i) bleomycin-induced double-strand cleavage exhibits strictly single-hit kinetics, (ii) the cleavages in both strands are based on C-4′ abstraction, (iii) the two abstraction

sites are 15-18 Å apart in B-DNA and (iv) there is no evidence that the drug is bifunctional, it has been concluded that the two breaks are probably made in succession by a single bleomycin molecule that is reactivated in the process of forming the first break [26-28]. There is some evidence that the presence of the first break may direct the putative repositioning of bleomycin molecule for attack at a specific site in the complementary strand [29]. The ultimate hydrogen-abstracting bleomycin species is still not known with certainty, but is believed to be a ternary bleomycin-iron-oxo complex, with iron in a high-valence Fe(IV) or Fe(V) state. As has now been verified by mass spectrometry [30], its immediate precursor, termed "activated bleomycin", is bleomycin ferric peroxide (HOO^-•Fe(III)•bleomycin).

Although the chemical synthesis of bleomycin was first reported in 1982, recent technical improvements in the synthesis have recently allowed creation of a much wider array of analogues for basic and clinical investigation [28,31].

3. Repair of DNA Double-strand Breaks

It is generally agreed that the cytotoxic effects of radiomimetic drugs are due primarily to DNA double-strand cleavage [2,3]. Although much of the basis for this belief derives from analogy with ionizing radiation, it is also supported by data showing that cells deficient in the end-joining pathway of double-strand break repair (see below) are hypersensitive to radiomimetic drugs [32-34]. As recently as five years ago, almost nothing was known about the biochemistry of DNA double-strand break repair in mammalian cells, but several of the critical factors involved have since been identified and their biochemical functions at least partially revealed.

In all eukaryotes there are two distinct modes of double-strand break repair, homologous recombination and nonhomologous end-joining. Homologous recombination involves borrowing a DNA strand from an intact second copy of the same sequence, and using that strand as a template to accurately restore the original sequence at the break site. Several genes required for this pathway have been identified, including *hRAD51*, which is homologous to *RAD51* of *Saccharomyces cerevisiae* and *recA* of *E. coli*. Like its homologues, the hRad51 protein catalyzes ATP-dependent DNA strand exchange [35]. The *XRCC2* and *XRCC3* genes were isolated on the basis of their ability to correct the radiosensitivity of certain rodent cells, and also have weak homology to *RAD51* [36]. The *BRCA1* and *BRCA2* gene products, identified on the basis of their association with hereditary predisposition to breast cancer, appear to colocalize with hRad51 in distinct, Xrcc3-dependent nuclear foci in irradiated cells, and thus may also be implicated, but their precise function(s), like those of Xrcc2 and Xrcc3, are not known [37,38].

Although simpler in concept, repair by nonhomologous end-joining appears to be operationally quite complex [39-42]. Genetic data have clearly implicated DNA-dependent protein kinase (DNA-PK), consisting of the 465-kDa catalytic subunit DNA-PKcs plus the 70- and 86-kDa Ku proteins, as playing an essential role in this repair pathway [43,44]. Repair is thought to begin with the assembly of DNA-PK on each DNA end (Fig. 3). The 70- and 86-kDa subunits of Ku protein probably bind first, and then recruit the catalytic subunit DNA-PKcs, whose kinase activity is thus activated [42,45,46]. Although the critical phosphorylation targets are not known, it has been concluded from inhibitor studies in a *Xenopus* system that alignment of the two DNA ends, as well as a DNA-PK-catalyzed phosphorylation event, must precede all biochemical processing of the termini [47,48], including removal of 3′-phosphoglycolates and presumably other terminal blocking groups. One possibility is that successful alignment of the two ends triggers phosphorylation of Ku86 and/or Ku70 (both of which are DNA-PK substrates *in vitro* [49]), resulting in either dissociation of Ku or sliding of Ku from the end into an interior

position on the DNA, and thus allowing repair enzymes access to the DNA end [40,47,48,50,51]. Trimming (possibly by the *FEN1* "flap endonuclease" [52]) and/or patching (probably by DNA polymerase β) then presumably generates a fully duplex DNA substrate that is continuous except for a single nick in each strand. The nicks are sealed by DNA ligase IV, with participation of the *XRCC4* gene product [53-55]; remarkably, this ligation can apparently proceed despite mismatches in annealed overhangs, as long as there is at least one complementary base pair between the mismatch and the terminus [56].

The question of whether homologous recombination or nonhomologous end-joining is more important in repair of double-strand breaks continues to be a matter of controversy. The radiation hypersensitivity of cells deficient in Ku or DNA-PKcs [39,40] has been taken as evidence that end-joining is the dominant pathway. However, the finding that overexpression of hRad51 confers radioresistance suggests a more complex relationship between the two pathways [57]. Perhaps the most likely possibility is that end-joining may be more important in the G1/G0 phase of the cell cycle, whereas homologous recombination may be more important in late S and G2 phase, when the replicated homologous daughter duplexes are in close physical proximity, and thus more readily available for strand exchange [58,59].

The Mre11 complex, consisting of hMre11, hRad50 and Nbs1 (the protein defective in Nijmegen [chromosome] breakage syndrome) also appears to play an important but not as yet clearly defined role in double-strand break repair. In yeast, deficiency in any component of this complex impairs both homologous and nonhomologous double-strand break repair pathways, but with a more severe effect on the latter [60]. Intriguingly, following irradiation of human cells, the Mre11 complex localizes to foci in regions of the nucleus that have sustained radiation damage [61]. However, it appears that any given cell in a population is competent to form either Mre11 foci or hRad51 foci (see above), but not both [62]. Cells deficient in Nbs1, like ataxia telangiectasia cells, fail to arrest DNA synthesis in response to irradiation, suggesting involvement of the Mre11/hRad50/Nbs1 complex in the recognition as well as the repair of double-strand breaks. Deficiency in hMre11 or hRad50 appears to be lethal [61].

The mechanism by which double-strand breaks ultimately effect cell killing remains a highly controversial and critically important topic, but one that is beyond the scope of this review. In recent years, the classical view of the cell being a passive victim of DNA damage has been downplayed in favor of a model in which the cell is an active effector of its own destruction. Following the recent identification of certain critical proteases (caspases), apoptotic pathways leading from initial DNA damage to programmed cell destruction have now been delineated in much detail [63,64], but the chemotherapeutic relevance of these pathways continues to be challenged, at least for nonhematopoietic tumors [65]. Harnessing programmed cell death pathways so as to selectively potentiate the toxicity of DNA damage in certain cells is an approach of great promise and intensive investigation. For the present review, however, we will confine the discussion to approaches which modulate toxicity by affecting levels of DNA damage or repair.

4. Clinical Applications and Toxicities of Naturally Occurring Compounds

Of the enediynes, Zinostatin (Kayaku) has gone through the most extensive preclinical and clinical evaluation. Animal studies with zinostatin were initially promising, but subsequent clinical trials showed that, as with most DNA-damaging drugs, the limiting toxicity was immunosuppression, occurring even at the lowest effective drug doses (reviewed by Maeda [66]). Because of a relatively low therapeutic index, the natural compound is today little-used.

There is limited usage of a styrene maleic acid conjugate of the drug (SMANCS), administered by intraarterial infusion, for treatment of hepatocellular carcinoma [67]. Other enediynes, despite activity against a number of tumor cell lines, have not shown sufficient therapeutic indices in animal studies to qualify for clinical trials [4,68].

Bleomycin, however, continues to be used extensively in clinical chemotherapy, most prominently in combination with adriamycin, vinblastine and dacarbazine (the ABVD protocol) for treatment of Hodgkins lymphoma [69]. Its primary advantage over earlier nitrogen mustard-based regimens is an apparently lower incidence of second cancers [70], which probably result from the carcinogenic effects of mustard-induced DNA damage. Use in non-Hodgkins lymphoma [71], and in squamous cell carcinomas of the head and neck [72] is also well-established. Evaluation of bleomycin in various combination chemotherapy protocols continues to be actively pursued, with about 50 reports of clinical trials appearing every year.

The primary reason for the popularity of bleomycin in combination chemotherapy is its unique spectrum of clinical toxicity; unlike most DNA-damaging drugs, bleomycin produces very little immunosuppression, apparently due to high levels of the inactivating enzyme bleomycin hydrolase in bone marrow (see Fig. 2) [73]. Homozygous bleomycin hydrolase-deficient (*Blmh*) knockout mice have recently been generated [74], and their profound sensitivity to the lethal effects of bleomycin confirms the importance of this enzyme in modulating cytotoxicity.

The limiting clinical toxicity of bleomycin is a severe and sometimes fatal pulmonary fibrosis, the incidence of which increases with cumulative bleomycin dose [75]. In fact, intratracheal bleomycin administration in rodents has become the standard model system for investigating mechanisms of human idiopathic pulmonary fibrosis. Studies with this rodent model have revealed that the bleomycin-induced fibrotic response is quite complex and involves numerous inter- and intracellular signaling factors, including interleukins IL-5 [76] and IL-6 [77], transforming growth factor α (TGFα) [78], TGFβ1 [79], tumor necrosis factor (TNF) [77,80], macrophage inflammatory protein 1α (MIP-1α) [77], integrin $\alpha v\beta 6$ [76] and granulocyte-macrophage colony stimulating factor (GM-CSF) [79]. Although there have been few attempts to exploit these findings clinically, they suggest that there may be considerable potential for ameliorating fibrosis, once the cytokine pathways are more well-defined. One antifibrotic agent, pirfenidone (5-methyl-1-phenyl-2-(1H)-pyridone) reduced bleomycin-induced pulmonary fibrosis in a hamster model [81] (perhaps in part by inhibiting procollagen gene expression [82]), and also had some activity against idiopathic pulmonary fibrosis in a phase II clinical trial [83]. At least in some early studies [84], pepleomycin (Fig. 2) seemed to produce somewhat less pulmonary toxicity than other bleomycins.

5. Approaches to Targeting and Modulating Cytotoxicity

5.1 Chemical Modulators

The aminothiol WR-2721 (amifostine; NH_2-$(CH_2)_3$-NH-$(CH_2)_2$-$SH_2PO_3 \bullet 1.5H_2O$), which is dephosphorylated *in vivo* by alkaline phosphatase to yield the corresponding biologically active free sulfhydryl form WR-1065, has emerged as the most promising of the many potential free radical scavengers that have been evaluated as radioprotectors [85]. It appears to accumulate to a greater extent in normal than in tumor tissue, and can react with electrophilic DNA-damaging agents such as cisplatin and cyclophosphamide, thus reducing many of the untoward side effects of these drugs (as well as those of radiation) without reducing therapeutic efficacy [86].

For radiomimetic antibiotics, the potential effects of these compounds are complex. While free aminothiols could conceivably quench either the activated forms of the antibiotics or the initial free radicals on deoxyribose in DNA (and thereby reduce DNA damage), these radicals may not be accessible to scavenger when formed within a tight antibiotic-DNA complex. Moreover, the thiols could also serve to increase sulfhydryl-dependent drug activation in the nucleus and thus enhance DNA damage; this is especially true for bleomycin, since by far the slowest step in its catalytic cycle is the reduction of Fe(III)•bleomycin to Fe(II)•bleomycin [25]. Thus, it is not surprising that, for various endpoints and cell lines, WR-1065 has been shown to produce both enhancement of [87] and protection from [88] bleomycin damage. Of particular interest, however, is the finding that amifostine seems to protect against bleomycin-induced pulmonary fibrosis [89], which may be triggered by free radical damage to targets other than DNA [90]. Thus, it may be possible to devise protocols using this or similar radioprotectors, that would selectively protect against lung toxicity while not reducing, and perhaps even enhancing, cytotoxicity to tumor cells.

5.2 Electrochemotherapy

With LD_{50} values for various tumor cell lines in the micromolar range, bleomycin is a much weaker cytotoxin than most of the enediynes. This relative lack of potency is due primarily to very poor uptake of the large, hydrophilic bleomycin molecule into cells. The mechanism of uptake is still poorly delineated, but it appears to be saturable and to involve initial drug binding at the cell surface followed by endocytosis [91]. Studies in cultured tumor cells showed that bleomycin-induced cell killing was enhanced more than 100-fold (to an LD_{50} of about 2 nM) when the cells were permeabilized by electroporation to facilitate drug uptake [92]. Thus, in theory, the intrinsically poor uptake of bleomycin could be used to advantage if tumor cells could be selectively permeabilized.

Preclinical studies, using human rhabdomyosarcoma xenografts in immunodeficient rats, showed that the effectiveness of bleomycin injected directly into tumor nodules was increased dramatically when the injection was followed within a few minutes by localized electroporation of the region containing the tumor [93]. Nearly half of the tumors were apparently cured by the bleomycin + electroporation treatment, while treatment with either bleomycin or electroporation alone had virtually no effect on tumor growth. There was little toxicity to nontumor tissue even in the electroporated area.

In a phase I clinical trial, a variety of subcutaneous tumor nodules were treated with systemic intravenous bleomycin, followed by electroporation, which consisted of several 100-μsec pulses of $\sim$1,000 volts/cm administered through electrodes placed on skin on opposite sides of the tumor nodules [94]. Significant responses were seen in all tumor types examined, including melanoma, adenocarcinoma, basal cell carcinoma, and head and neck squamous cell carcinoma. As in the animal studies, the treatment was remarkably well-tolerated by normal tissue.

5.3 Drug-antibody Conjugates

The concept of a "magic bullet", consisting of a potent cytotoxin conjugated to a ligand molecule that would target the toxin to tumor cells, has been pursued for many years, thus far with relatively little clinical success. Attempts to use antibodies as the targeting moieties have been complicated by such factors as antigenicity of the antibody, reduced potency of the cytotoxin upon conjugation, poor penetration of the conjugate into the tumor, and emergence of resistant, antigen-negative subpopulations of cells within the tumor over the course of therapy

[95]. The use of antibody fragments and recombinant chimeric antibodies containing human constant regions has reduced antigenicity problems [96], but there are still few toxins that are sufficiently potent that an antibody conjugate, even if successfully targeted, will accumulate in tumor cells in sufficient quantity to effect cell killing.

Zinostatin is in theory an attractive agent for conjugation because it consists of a small aromatic DNA-cleaving "chromophore" bound tightly but noncovalently to a 109-amino acid protein which protects the highly labile chromophore from spontaneous degradation in aqueous solution [2,97]. Thus, it is possible that zinostatin linked through its protein constituent to an antibody or other targeting species would bind to the cell surface and allow the chromophore to be taken up into the cell, without requiring either internalization or intracellular cleavage of the conjugate. Preclinical studies with zinostatin conjugates have centered on the A7 mouse monoclonal antibody, which recognizes an as yet unidentified surface antigen present on cells of many human pancreatic, gastric, and colorectal carcinomas. A zinostatin-A7 conjugate was found to be fourfold more toxic than zinostatin itself to antigen-positive MKN45 gastric carcinoma cells grown in culture [98]. The enhanced toxicity appeared to be due to conjugate binding to the A7 antigen, since the conjugate was not more toxic than the parent compound to antigen-negative MKN1 cells. The zinostatin-A7 conjugate was also more effective than the parent compound in suppressing formation of intraperitoneal nodules in nude mice inoculated with MKN45 (A7 antigen-positive) cells.

In order to reduce antigenicity and improve tumor penetration, zinostatin conjugates were prepared using either proteolytic Fab fragments of the A7 antibody (in which a large portion of the heavy chain constant region has been deleted), or recombinant chimeric Fab fragments containing the antigen-binding variable region from the mouse antibody, and a human constant region. Preliminary studies in nude mice showed that while accumulation of Fab fragments in HPC-YS xenografts (a human pancreatic carcinoma, positive for A7 antigen) was slightly less than that of the full A7 antibody, the clearance of the Fab fragment was so much faster that the ratio of levels in the tumor to levels in the plasma was tenfold higher for the Fab fragment than for the original A7 antibody [99]. In addition, the chimeric Fab fragment was at least 600 times less antigenic than the original A7 antibody, as assessed by its reactivity with rabbit anti-mouse IgG serum. Subsequent studies with a chimeric Fab fragment conjugated to zinostatin showed that it was more effective than the original zinostatin-A7 conjugate (and much more effective than native zinostatin) in suppressing growth of HPC-YS xenografts [100].

Calicheamicin, because of its extreme cytotoxicity, has also been the subject of several attempts to construct antibody conjugates. Linkage is typically through the trisulfide (shortened to a disulfide in the conjugates), so that release of calicheamicin from the conjugate by reduction of the disulfide simultaneously activates the drug for DNA cleavage (see Fig. 1). A series of such agents was prepared using mouse monoclonal antibody CT-M-01, an internalizing antibody that binds to a human cell surface mucin known to be present in a number of solid tumors [101]. The antibody was linked to each of several calicheamicins through the trisulfide moiety, and the various conjugates were assessed for activity against human MX-1 breast carcinoma, which carries the target mucin. For XC-RM rat sarcoma cells, which lack the target mucin, the γ^I_1 antibody conjugate was at least 20-fold less toxic than calicheamicin γ^I_1 itself, but for the antigen-positive MX-1 cells the conjugate was as toxic as the parent compound, suggesting that the conjugate was effectively targeted. Moreover, antibody conjugates of several calicheamicins (including α^I_3 and three forms of γ^I_1 with slightly different linkers), effectively arrested growth of MX-1 xenografts in athymic mice at well-tolerated doses; in contrast, all of the parent calicheamicins were lethal to the animals at doses that had little or no effect on tumor growth.

More recently, total synthesis of calicheamicin has yielded calicheamicin Θ^I_1, which is more stable and, with lethal doses in the low femtomolar range, even more cytotoxic than the natural calicheamicins. Calicheamicin Θ^I_1 was conjugated to mouse monoclonal antibody 14G2a, which recognizes the murine GD_2 ganglioside present in normal neuroectodermal tissue and in many neuroblastomas. Even though the conjugate was 100-fold less toxic to the target NXS2 murine neuroblastoma cells than was calicheamicin Θ^I_1 itself, the conjugate was, because of its greater selectivity, much more effective than the parent drug in suppressing liver metastases in nude mice inoculated with NXS2 cells [102]. Thus, while the results support the feasibility of targeted chemotherapy, they also emphasize the need to begin with a parent compound of very potent intrinsic cytotoxicity. The results are encouraging in that (unlike results from xenograft models) they show that a significant improvement in therapeutic index can be achieved even with a target tumor antigen that is also expressed on some normal cells.

CD33 is an antigen that is present on most normal human myeloid hematopoietic cells and on many acute myeloid leukemia cells, but is absent from normal hematopoietic stem cells and from all nonhematopoietic cells. Antibodies recognizing CD33 have been conjugated to several radionuclides, including ^{131}I and ^{237}Bi, and have been used in attempts at targeted radiotherapy [103]. A phase I clinical trial using calicheamicin γ^I_1 conjugated to a chimeric human/mouse anti-CD33 antibody showed some promise in patients with relapsed or refractory acute myeloid leukemia [104]. The conjugate was well-tolerated at doses sufficient to saturate available cellular binding sites, and a few patients recovered normal blood cell counts. A phase II trial is in progress.

5.4 Repair Inhibitors

In theory, it should be possible to increase the cytotoxicity of both bleomycin and the enediynes by inhibiting essential components of double-strand break repair pathways. DNA-PK is an attractive target in this regard, because a DNA-PK-catalyzed phosphorylation event appears to be rate-limiting in the repair of terminally blocked double-strand breaks (Fig. 3) [48], and because DNA-PK deficiency seems to have relatively little deleterious effect on cells [33,34,105] or knockout mice [106] other than hypersensitivity to agents that induce double-strand breaks, and lack of V(D)J recombination (which is likewise attributable to a defect in DNA end-joining). In preliminary cellular studies, the most frequently used DNA-PK inhibitor has been wortmannin, a fungal toxin that binds covalently to DNA-PK at the ATP binding site, completely inactivating its kinase activity [107]. Wortmannin inhibits phosphatidylinositol 3-kinase (PIK) and several PIK-related protein serine-threonine kinases, including DNA-PK and Atm (product of the *ATM* gene defective in ataxia telangiectasia), but it has no known effect on other, non-PIK-related protein kinases [108,109]. Despite this broad specificity, it was found that transient exposure to noncytotoxic concentrations of wortmannin could inhibit double-strand break repair and sensitize cultured cells to radiation and bleomycin [110-112]. Wortmannin-induced radiosensitization was seen in Atm-deficient but not in DNA-PK-deficient cells, suggesting that DNA-PK rather than Atm was the critical target [110].

Thus, inhibition of DNA-PK may have considerable potential as a mechanism for enhancing cytotoxicity of radiomimetic drugs. However, before this approach can even be assessed for practical utility, more specific DNA-PK inhibitors will have to be found, and ways of effectively targeting them to tumor cells may have to be devised.

6. Conclusions

The chemical mechanisms by which radiomimetic natural products such as bleomycin, zinostatin, esperamicin and calicheamicin induce double-strand breaks in DNA are now known in great detail, but bleomycin remains the only agent in this group that has found extensive use in clinical therapy. Incremental improvements in its use may be obtained by continued optimization of combination chemotherapy protocols, and perhaps by the newer technique of local electropermeabilization. However, it must be said that there have in recent years been few major advances in clinical use of radiomimetic agents, and the failure of any of the natural enediynes to enter mainstream chemotherapy has been particularly disappointing. Nevertheless, there have been several breakthroughs in basic and preclinical studies, including the complete chemical synthesis of bleomycin and calicheamicin, the cloning of the bleomycin-inactivating enzyme bleomycin hydrolase, the development of functional calicheamicin-antibody conjugates, and the identification and cloning of several double-strand break repair proteins that may be potential targets for enhancing cytotoxicity of radiomimetic drugs. Thus, there appears to be reason for optimism that at least some of these approaches will significantly increase the clinical utility of these agents over the next several years.

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Correspondence:
Dr. Lawrence F. Povirk
Medical College of Virginia
P.O. Box 980230
Richmond, VA 23298 U.S.A.
(804) 828-9640 / 828-8079FAX
LPOVIRK@gems.vcu.edu

TABLE I. CHARACTERISTICS OF NATURALLY OCCURRING ENEDIYNES

Drug [ref.]	3′ Stagger	Diradical[a]	Other Structural Features	Bistranded Lesions (%)	Activation
Neocarzinostatin (Zinostatin) [2,5,6]	2 bp	F	naphthoate, aminosugar, cyclic carbonate	20	Sulfhydryls, $NaBH_4$
C-1027 [7,8]	2 bp	F		50	Spontaneous
Kedarcidin [9]	n.d.[b]	F	naphthoate, aminosugar, macrocyclic ring	≥ 10[c]	Sulfhydryls
Dynemicin [10,11]	3 bp	B	anthraquinone, no sugars	Low[c]	NADPH, Light
Calicheamicin [12,13]	3 bp	B	trisulfide, aminosugars	98	Sulfhydryls
Esperamicin [14,15]	3 bp	B	trisulfide, aminosugars	≥ 10[c] (Esp A) ≥ 60[c] (Esp C)	Sulfhydryls

a. Zinostatin-like fused ring (F) or benzenoid (B)

b. Not determined

c. Estimated from visual inspection of published electrophoresis data. Since abasic sites may not have been cleaved in these experiments, the values given are probably minimum estimates.

FIGURE LEGENDS

Figure 1. Structure of calicheamicin γ^{I}_{1} [12], and its activation by sulfhydryl. For the activated form, only a partial structure is shown.

Figure 2. Structure of bleomycin showing (1) double bond that is a single bond in phleomycin and (2) β-aminoalanine that is deaminated by bleomycin hydrolase. The common clinical formulation Blenoxane (Bristol) contains primarily bleomycins A_2 and B_2 plus lesser amounts of several species with other t
terminal amine moieties.

Figure 3. Proposed model for the repair of terminally blocked double-strand breaks by the nonhomologous end-joining pathway. Binding of Ku and DNA-PKcs is followed by recruitment of additional proteins, alignment of the ends (possibly aided by as-yet-unidentified alignment protein(s)), and DNA-PK-catalyzed phosphorylation of one or more proteins in the complex, probably including Ku. Biochemical processing of the termini can then proceed, including removal of damaged termini, trimming of unpaired nucleotides, fill-in of any gaps and ligation by DNA ligase IV plus Xrcc4.

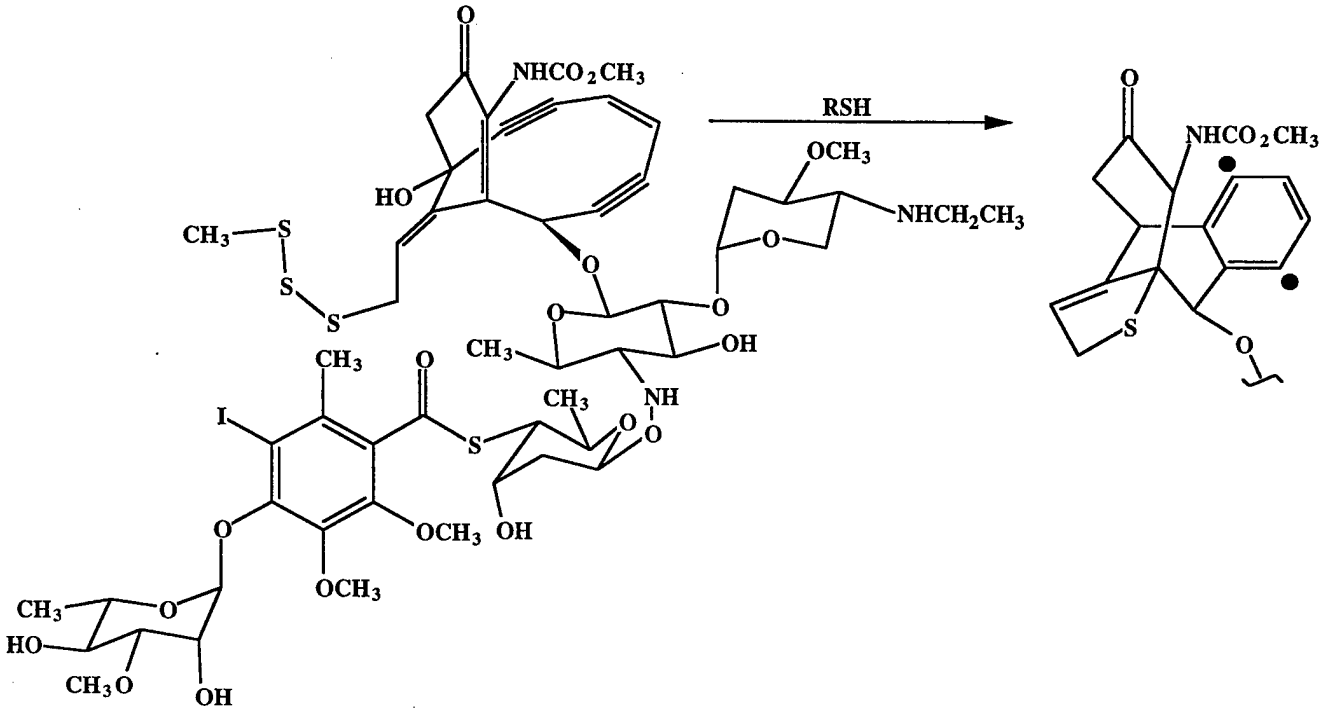

Figure 1

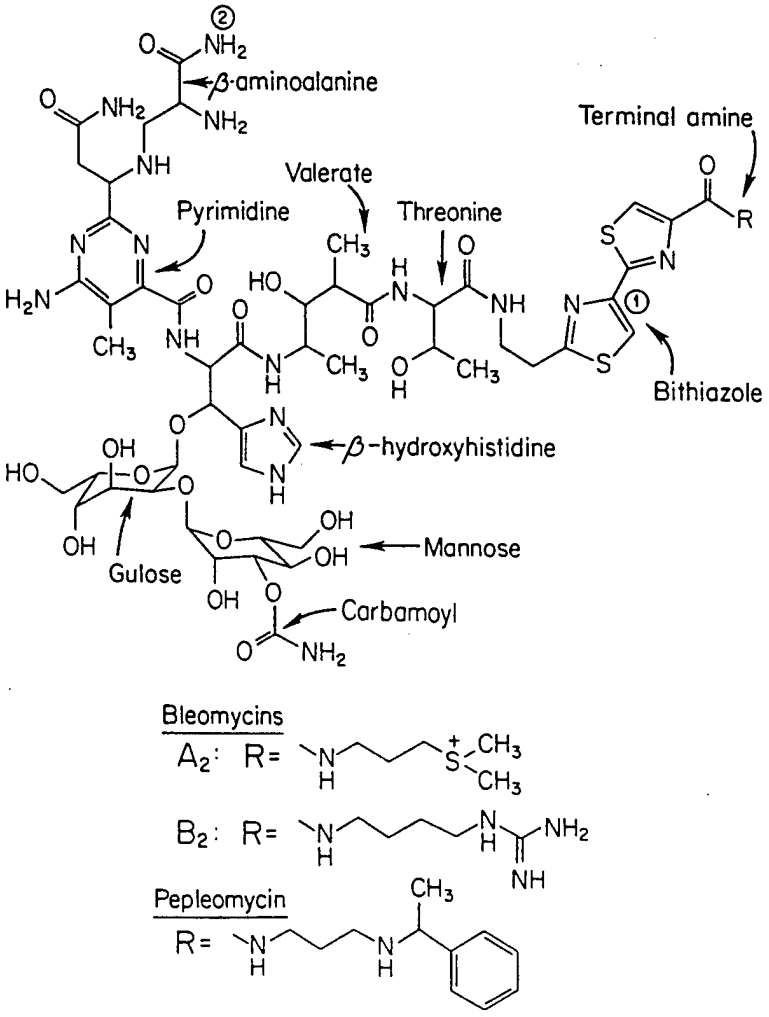
β-aminoalanine
Terminal amine
Valerate
Pyrimidine
Threonine
Bithiazole
β-hydroxyhistidine
Mannose
Gulose
Carbamoyl
Bleomycins
A2: R=
B2: R=
Pepleomycin
R=

Figure 2

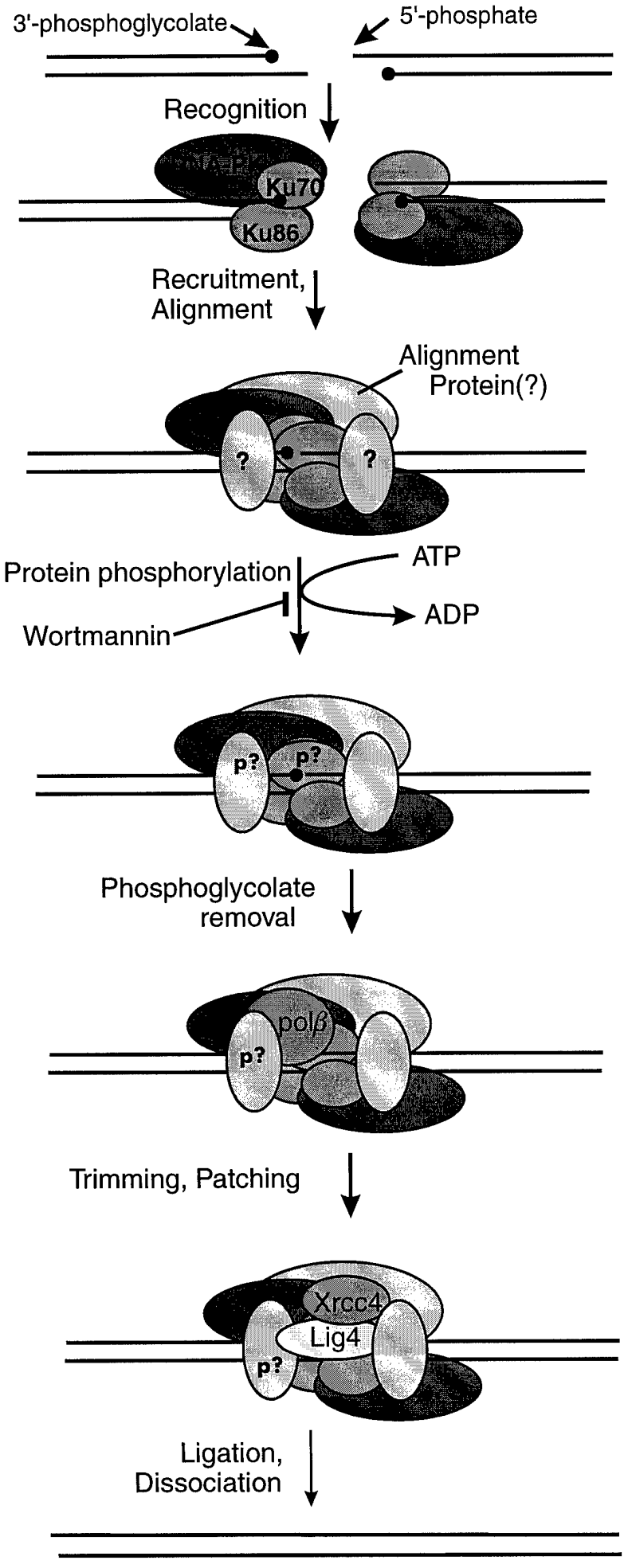
3'-phosphoglycolate
5'-phosphate
Recognition
DNA-PK
Ku70
Ku86
Recruitment,
Alignment
Alignment
Protein(?)
?
?
Protein phosphorylation
ATP
ADP
Wortmannin
p?
p?
Phosphoglycolate
removal
polβ
p?
Trimming, Patching
Xrcc4
Lig4
p?
Ligation,
Dissociation

Figure 3

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Inhibition of Protein Kinase C Activator-Mediated Induction of $p21^{CIP1}$ and $p27^{KIP1}$ by Deoxycytidine Analogs in Human Leukemia Cells

RELATIONSHIP TO APOPTOSIS AND DIFFERENTIATION

Julie A. Vrana, Lora B. Kramer,* Aida M. Saunders,* Xiao-Fan Zhang,† Paul Dent,‡ Lawrence F. Povirk† and Steven Grant*†§*‖

DEPARTMENTS OF *MEDICINE, §MICROBIOLOGY, †PHARMACOLOGY, AND ‡RADIATION ONCOLOGY, MEDICAL COLLEGE OF VIRGINIA, RICHMOND, VA 23298-0230, U.S.A.

ABSTRACT. Events accompanying sequential exposure of U937 leukemic cells to the deoxycytidine (dCyd) analogs 1-[β-D-arabinofuranosyl]cytosine (ara-C) or 2′,2′-difluorodeoxycytidine (gemcitabine; dFdC) followed by two protein kinase C (PKC) activators [bryostatin 1 (BRY) or phorbol 12′-myristate 13′-acetate (PMA)] exhibiting disparate differentiation-inducing abilities were characterized. A 24-hr exposure to 10 nM BRY or PMA after a 6-hr incubation with 1 μM ara-C or 100 nM dFdC resulted in equivalent increases in apoptosis, caspase-3 activation, and polyADP-ribose polymerase degradation, as well as identical DNA cleavage patterns. BRY and PMA did not modify retention of the lethal ara-C metabolite ara-CTP or alter ara-CTP/dCTP ratios. Unexpectedly, pretreatment of cells with ara-C or dFdC opposed BRY- and PMA-related induction of the cyclin-dependent kinase inhibitors (CDKIs) $p21^{CIP1}$ and/or $p27^{KIP1}$. These effects were not mimicked by the DNA polymerase inhibitor aphidicolin or by VP-16, a potent inducer of apoptosis. Inhibition of PKC activator-induced CDKI expression by ara-C and dFdC did not lead to redistribution of proliferating cell nuclear antigen but was accompanied by sub-additive or antagonistic effects on leukemic cell differentiation. Sequential exposure of cells to ara-C followed by BRY or PMA led to substantial reductions in clonogenicity that could not be attributed solely to apoptosis. Finally, pretreatment of cells with ara-C attenuated PMA- and BRY-mediated activation of mitogen-activated protein kinase, an enzyme implicated in CDKI induction. Collectively, these findings suggest that pretreatment of leukemic cells with certain dCyd analogs interferes with CDKI induction by the PKC activators PMA and BRY, and that this action may contribute to modulation of apoptosis and differentiation in cells exposed sequentially to these agents. BIOCHEM PHARMACOL 58;1:121–131, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. ara-C; gemcitabine; bryostatin; PMA; apoptosis; differentiation; $p21^{CIP1}$; $p27^{KIP1}$; U937 cells

In hematopoietic cell systems, a complex relationship exists between apoptosis and differentiation. For example, leukemic cells undergoing terminal differentiation in response to retinoic acid [1] or PMA¶ [2] ultimately undergo an apoptotic form of cell death. In contrast, differentiation induction has been shown to block apoptosis in response to diverse stimuli, including cytokines (transforming growth factor β) [3], proteasome inhibitors (lactacystin) [4], and cytotoxic drugs (e.g. etoposide) [5]. Moreover, leukemic cells displaying dysregulation of the Ca^{2+}- and lipid-dependent serine-threonine kinase PKC undergo apoptosis rather than maturation when exposed to PMA [6]. Collectively, such findings raise the possibility that apoptosis represents an alternative fate for cells unable to proceed along a normal differentiation pathway [7].

There is evidence that under appropriate conditions, induction of cellular maturation potentiates apoptosis in leukemic cells previously exposed to cytotoxic agents. For example, differentiating compounds such as vitamin D_3, DMSO, hexamethylamine bisacetamide, all-*trans* retinoic acid, and *n*-butyrate have been shown to enhance leukemic cell apoptosis following exposure to DNA-damaging drugs including ara-C, 5-fluorouracil, and camptothecin, among others [8–11]. Although the mechanism underlying this phenomenon is unknown, it has been hypothesized that induction of maturation interferes with DNA repair events that accompany genotoxic insults [12]. An alternative possibility, consistent with the findings cited above, is that drug-mediated DNA damage leads to a disruption of normal differentiation events, signaling the cell to proceed along an apoptotic pathway.

‖ Corresponding author: Dr. Steven Grant, Medical College of Virginia, MED-HEM/ONC, Box 980230 MCV Station, Richmond, VA 23298-0230. Tel. (804) 828-5211; FAX (804) 225-3788.

¶ *Abbreviations:* PMA, phorbol 12′-myristate 13′-acetate; PKC, protein kinase C; ara-C, 1-[β-D-arabinofuranosyl]cytosine; dFdC, (gemcitabine), 2′,2′-difluorodeoxycytidine; BRY, bryostatin 1; CDKIs, cyclin-dependent kinase inhibitors; dCyd, deoxycytidine; PCNA, proliferating cell nuclear antigen; MAPK, mitogen-activated protein kinase; PARP, polyADP-ribose polymerase; PCR, polymerase chain reaction; and APC, aphidicolin.

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BRY is a non-tumor promoting PKC activator that exerts variable effects on leukemic cell differentiation [13] and exhibits antitumor activity in non-hematological malignancies as well [14]. It represents one of a group of natural products targeted by the National Cancer Institute for clinical development in humans [15]. In human monocytic leukemic cells (U937), BRY, administered at high concentrations (e.g. 200 nM), inhibits cell growth while inducing dephosphorylation of the cyclin-dependent kinase CDK2 [16], although several studies have shown that its capacity to inhibit proliferation in these cells is considerably weaker than that of PMA [17–19]. Recently, our laboratory undertook a direct comparison of BRY and PMA (10 nM each) with respect to their effects on cell cycle regulatory proteins and induction of differentiation in U937 cells [20]. This study demonstrated that BRY is considerably less potent than PMA in inducing $p21^{CIP1}$, inhibiting CDK2 activity, and triggering cell cycle arrest and growth inhibition, providing a possible mechanism for its limited differentiation-inducing capacity in this cell line.

In a previous paper, we reported that administration of BRY before, but not after ara-C in differentiation-unresponsive human leukemia cells (HL-60) leads to an increase in apoptosis accompanied by synergistic inhibition of clonogenicity [21]. In contrast, in weakly differentiation-responsive U937 leukemic cells, apoptosis is only potentiated when BRY follows ara-C [19]. Thus, in this system, BRY may function like a differentiation inducer in promoting apoptosis in cells previously exposed to a DNA-damaging agent [8–11]. Currently, virtually no information exists concerning the mechanism by which maturation-inducing compounds potentiate apoptosis in cells previously exposed to cytotoxic agents, nor have the events accompanying this phenomenon been well characterized. The purpose of the present study was to compare two PKC activators with disparate differentiation-inducing capacities (i.e. BRY and PMA) with respect to their effects on drug-induced apoptosis in U937 cells, and to identify factors that might be involved in modulating apoptosis and maturation in cells pre-exposed to two cytotoxic dCyd analogs, ara-C and dFdC. The potential relevance of this comparison is highlighted by a very recent report demonstrating the feasibility of administering PMA to patients with leukemia, as well as possible beneficial *in vivo* interactions of this agent with ara-C [22]. Our findings indicate that BRY and PMA exerted qualitatively and quantitatively similar effects on dCyd analog-mediated apoptosis; moreover, pretreatment of cells with ara-C or dFdC blocked PKC activator-associated induction of the (CDKIs) $p21^{CIP1}$ and/or $p27^{KIP1}$, a phenomenon associated with disruption of cellular maturation. These observations raise the possibility that dysregulation of the cell cycle arrest machinery may contribute to apoptotic and differentiation-related responses observed in cells sequentially exposed to ara-C or dFdC followed by PKC-activating agents.

MATERIALS AND METHODS

Cells

The human monocytic leukemic cell line U937 was derived from a cell line as previously reported [23]. Cells were cultured to logarithmic growth phase in RPMI 1640 medium supplemented with sodium pyruvate, MEM essential vitamins, L-glutamate, penicillin, streptomycin, and 10% heat-inactivated fetal bovine serum (HyClone). Cells were checked routinely and determined to be mycoplasma-free using the Gen-Probe Kit (Gen-Probe Inc.).

Drug Treatment

Logarithmically growing cells were exposed to ara-C (free base), APC, etposide (Sigma), or dFdC (provided by Dr. M. H. Niedenthal, Eli Lilly) for 6 hr, after which the cells were washed three times in serum-free medium to remove drug, and resuspended in medium containing BRY (provided by Dr. A. J. Murgo, CTEP/DCT) or PMA (Sigma) for an additional 24 or 72 hr. Cell number was determined by hemacytometer and normalized prior to the studies of apoptosis, differentiation, and protein expression, as described below. Vehicle controls of water and DMSO (≤0.01%) were found consistently to be equivalent to drug-free controls with respect to gene expression and apoptosis.

Differentiation and Apoptosis Studies

CD11b expression was evaluated after 72 hr of treatment as previously described [20, 24]. Briefly, treated cells were pelleted at 500 *g* and resuspended in cold PBS at 5×10^5 cells/mL. The cell suspension was mixed with fluorescein isothiocyanate-labeled antibody (CD11b or IgG2a control, Becton-Dickinson) and placed on ice for 20 min. Cells were diluted in cold PBS and analyzed with a Becton-Dickinson FACScan flow cytometer and CyCLOPS 2000 Version 4.0 software. Cell morphology and apoptosis were measured by cytocentrifuge preparations stained with the Diff-Quik stain set (Dade Diagnostics) and viewed by light microscopy. Features of cellular differentiation, as well as apoptosis (i.e. cell shrinkage, nuclear condensation, formation of apoptotic bodies) were evaluated as previously described [21]. The percentage of apoptotic cells was determined by evaluating ≥1000 cells per condition in three separate experiments.

Analysis of DNA Damage

To assess DNA fragmentation, pelleted cells (3×10^6 cells/pellet in triplicate) were resuspended in 0.5 mL of PBS and lysed by the addition of 5 mM Tris-HCl, 30 mM EGTA, 30 mM EDTA, 0.1% Triton X-100 (fully reduced), with gentle agitation. The lysates were centrifuged at 20,000 *g* at 4° for 40 min, the pellets were discarded, and the presence of DNA fragments in the supernatant was determined. Following treatment with RNase (50 μg/mL) for 2 hr at 37°, DNA from 3×10^5 cells was electropho-

resed on a 2.5% low-melting point agarose gel. Bands corresponding to mononucleosomal and dinucleosomal fragments were removed from the gel, and the DNA was recovered by β-agarase digestion (Epicentre Technologies) followed by ethanol precipitation. These DNA samples, as well as samples of genomic DNA that had been either sonicated and similarly gel-fractionated, or cut with *Alu*I, *Mbo*I, *Nla*III, *Tsp*509I, or *Sma*I, were Klenow treated to produce blunt ends and phosphorylated with polynucleotide kinase [25]. The DNA was ligated to the partial duplex GCGGTGACCCGGGAGATCTGAATTC-GAATTCAGATC [26], and amplified by anchored PCR, using the same 25-mer primer and primer CTCTGTCIC-CCAGGCTGGAGTGCA, corresponding to bases 268–245 of the minus strand of the *Alu* consensus sequence [27], for 25 cycles of 30 sec at 94°, 30 sec at 55° and 30 sec at 72° [28]. Following removal of primers by precipitation, the PCR products were subjected to runoff polymerization with the 5′-end-labeled, nested *Alu* primer CCCAGGCTG-GAGTGCAITGG (minus strand bases 260–241) using an Epicenter Epicycle sequencing kit but substituting a mixture containing 0.25 mM of each dNTP in place of the termination mixtures. Samples were electrophoresed for 4 hr on a 7% polyacrylamide gel and subjected to autoradiography. Cleavage positions were assigned to each band by reference to the restriction enzyme-treated samples, which yielded the expected prominent bands corresponding to site-specific cleavage of the *Alu* sequence.

Determination of ara-CTP Levels by HPLC

After treatment, cells were counted and equal numbers (20×10^6) were washed in cold PBS, lysed in 0.6 N trichloroacetic acid, and extracted in 1:3.5 trioctylamine: 1,1,2-trichlorotrifluoroethane (Sigma-Aldrich). The aqueous phase was stored at −80° until analysis. Immediately prior to column addition, the samples were thawed and extracted in succession with 0.5 M sodium periodate, 4 M methylamine, and 1 M rhamnose to convert the NTPs to their respective bases [29]. Extracts were run on a Waters radial-pak 10 μm SAX cartridge, monitored at 280 nm on a Beckman 160 detector, and analyzed with a Bio-Rad model 700 Chromatography Workstation (Version 3.63 software). Samples were run at 3 mL/min for 22 min in 25% ammonium phosphate (0.75 M, pH 3.7)–75% ammonium phosphate (5 mM, pH 2.8), which then was increased to 100% of the 0.75 M ammonium phosphate over the next 40 min [30]. Peaks were identified by relative retention time compared with authentic ara-C triphosphate and deoxycytidine triphosphate (Sigma).

Western Analysis

Cells were washed two times with PBS, resuspended in 100 μL PBS, lysed by the addition of 100 μL of 2× Laemmli buffer [60 mM Tris (pH 6.8), 4% SDS, 5.76 mM β-mercaptoethanol, 10% glycerol] and briefly sonicated. Lysates were quantified using Coomassie protein assay reagent (Pierce). Extracts (25 μg) were boiled for 10 min, fractionated by SDS–PAGE, transferred electrophoretically to Optitran nitrocellulose filters (Schleicher & Schuell), and probed with antibodies for $p21^{CIP1}$ and $p27^{KIP1}$ (1:500 dilution; Transduction Laboratories, Lexington). Detergent-insoluble and -soluble cell extracts for determination of PCNA were prepared exactly as described [31]. PCNA antibody was used at 1:500 (Upstate Biotechnology). After blocking in PBS-Tween (PBS-T; 0.05%) and 5% milk (Carnation) for 1 hr at room temperature, the membranes were incubated in fresh blocking solution with primary antibody for 4 hr at room temperature or overnight at 4°. Blots were washed subsequently three times for 10 min each in PBS-T, incubated for 1 hr with horseradish peroxidase-conjugated secondary antibody (Kirkegaard & Perry Laboratories) in blocking buffer, and washed three times for 10 min each in PBS-T. All blots were developed with the enhanced chemiluminescence method (Amersham). Equal gel loading and transfer were confirmed by staining the membrane with amido black after transfer and reprobing the blots with either anti-actin (Sigma) or anti-α-tubulin antibodies (Calbiochem).

Clonogenic Assay

Following drug treatment, cell number was determined by hemacytometer counting, and cells were washed three times in drug-free medium. Their ability to form colonies in soft agar was determined by a previously described technique [32]. Colonies, consisting of groups of ≥50 cells, were scored at day 10 utilizing an Olympus (Melville) model CK inverted microscope.

Determination of MAPK Activities

MAPK activity was determined as described previously [33]. Briefly, pelleted cells were washed in cold PBS, repelleted, and flash-frozen. MAPK was immunoprecipitated from clarified lysates with protein A/agarose-conjugated antibody/antisera (anti-ERK2, sc-154AC). Activity assay mixtures consisted of immunoprecipitated enzyme, substrate [myelin basic protein (Sigma)], and [γ-^{32}P]ATP (5000 Ci/pmol; NEN/DuPont), in 25 mM HEPES, pH 7.4, containing 15 mM $MgCl_2$, 100 mM trisodium orthovanadate, 0.01% β-mercaptoethanol, and 1 μM microcystin LR. Reactions were initiated by the addition of substrate and terminated by transfer to P81 filter paper. Filters were rinsed in 185 mM orthophosphoric acid, dehydrated in acetone, and then radioactivity was determined by liquid scintillometry.

Statistical Analysis

The significance of differences between experimental groups was determined utilizing Student's *t*-test for unpaired observations.

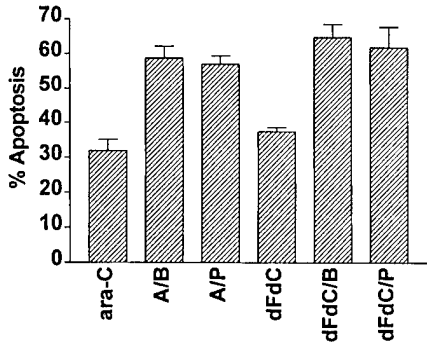
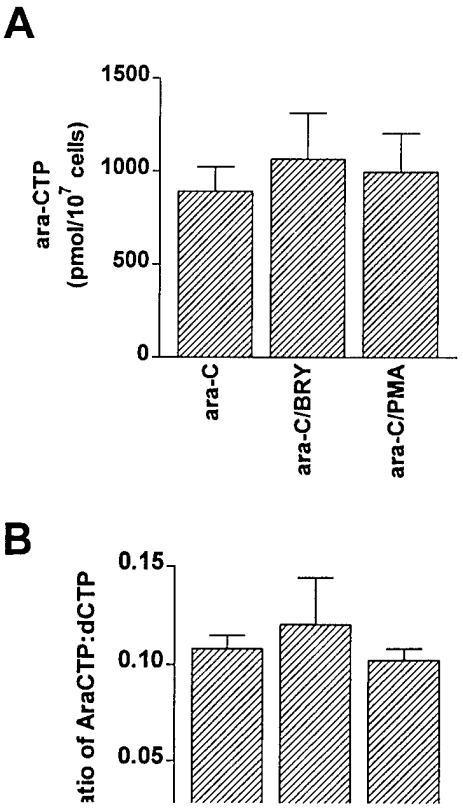


FIG. 1. Potentiation of ara-C- and dFdC-induced apoptosis by BRY or PMA. U937 cells at 4 × 10^5 cells/mL were pretreated with ara-C (1 μM) or dFdC (100 nM) for 6 hr, washed, and further incubated at 2 × 10^5 cells/mL with either medium, BRY (B) (10 nM), or PMA (P) (10 nM) for an additional 24 hr. The percentage of cells undergoing apoptosis was then assessed by monitoring Wright-Giemsa stained specimens for characteristic morphologic features as described in Materials and Methods. Values from triplicate experiments are expressed as the mean percent apoptosis ± SEM. Basal levels of apoptosis were less than 5%, and BRY and PMA treatment alone induced cell death in 8–10% of the cell population.



RESULTS

It has been shown in a previous report that exposure to BRY augments apoptosis in U937 cells previously treated with 10 or 100 μM ara-C [19]. However, the very extensive degree of cell destruction that resulted from such high ara-C concentrations, particularly in cells exposed subsequently to BRY or PMA, rendered analysis of accompanying events impractical. Consequently, for the purpose of the present study, a lower ara-C concentration (e.g. 1 μM) was employed. Whereas the extent of apoptosis (and its potentiation by BRY or PMA) was reduced thereby, this approach provided the major advantage of allowing associated events (e.g. expression of CDKIs and assessment of ara-C metabolism) to be monitored reliably.

The initial question to be addressed was whether the disparate abilities of PMA and BRY to induce U937 cell maturation [17, 18, 20] would have an impact on their capacity to modulate apoptosis in cells pre-exposed to ara-C (1 μM; 6 hr). Parallel studies were performed using equitoxic concentrations of the dCyd analog dFdC (e.g. 100 nM). In each case, drug concentrations were selected that induced apoptosis in ~35–40% of cells. Results are shown in Fig. 1. It can be appreciated that despite clear differences in their maturation-inducing ability, BRY and PMA produced equivalent increases in apoptosis in ara-C- and dFdC-pretreated cells (e.g. ~50–60% greater than that observed in cells exposed to dCyd analogs alone). It should be noted that a 24-hr exposure to BRY or PMA alone induced apoptosis to a limited degree (e.g. ~6–10%), and that in all cases the extent of apoptosis following sequential drug treatment was greater than additive ($P < 0.02$ in each case; not shown). Assessment of DNA fragmentation by bisbenzimide spectrofluorophotometry yielded equivalent results (not shown). Thus, despite its relatively weak differentiation-inducing capacity, BRY was as effective as PMA in triggering the characteristic morphologic features of apoptosis in ara-C- and dFdC-pretreated cells, suggesting that factors other than, or in addition to, differentiation *per se* were responsible for this phenomenon.

To determine whether BRY or PMA might act by modulating ara-C metabolism, the retention of the lethal ara-C derivative ara-CTP was compared in ara-C-pretreated cells subsequently incubated for 24 hr in either fresh medium or medium containing either 10 nM BRY or PMA (Fig. 2). It can be seen that subsequent exposure of cells to BRY or PMA did not increase ara-CTP retention significantly (Fig. 2A), nor did it alter the ratios of ara-CTP to its physiologic analog, dCTP ($P = 0.68$) (Fig. 2B). Consequently, potentiation of ara-C metabolism could not be

invoked to explain modulation of ara-C-mediated apoptosis by PKC activators. As we have reported previously, treatment of cells with PMA alone resulted in a modest arrest of cells in G_1 at 24 hr, whereas BRY has only a marginal effect [20]. When cells were exposed to ara-C or dFdC alone, an increase in the subdiploid fraction was observed, accompanied by a substantial reduction in the S-phase fraction (i.e. from ~40% in controls to 0.8 ± 0.4 and 8.0 ± 3.5% in the case of ara-C and dFdC, respectively; data not shown). When cells were exposed sequentially to the dCyd analogs followed by PMA or BRY, a further increase in the subdiploid fraction was noted, which occurred at the expense of the G_0/G_1, and, to a lesser extent, the G_2M fractions (data not shown). Reductions in the S-phase fractions under these conditions were equivalent to those seen in cells exposed to ara-C or dFdC alone. Thus, we were unable to attribute the observed increase in apoptosis in cells subsequently exposed to PMA or BRY to an expansion of the S-phase population.

To determine whether subsequent exposure of ara-C-pretreated cells to BRY or PMA produced qualitative differences in DNA fragmentation, mononucleosomal and dinucleosomal fragments were isolated from treated cells, and the positions of the double-strand breaks by which they were generated were mapped by ligation-mediated PCR in the highly repeated *Alu* sequence. In any defined DNA sequence, endonucleases typically exhibit very large site-to-site variation in cleavage frequency, resulting in a complex cleavage signature that is distinct for each nuclease (e.g. DNAse I [34], or the mammalian Ca^{2+}/Mg^{2+}-dependent nuclear endonuclease [35]). Thus, the finding that cleavage patterns in the *Alu* sequence were essentially identical for cells treated with ara-C either alone or followed by BRY or PMA (Fig. 3) suggests that the same endonuclease(s) was activated in each case. In separate studies, the extent of caspase-3 and PARP cleavage was equivalent in cells exposed to ara-C followed by either BRY or PMA (not shown). Moreover, caspase-3 activity increased to a similar extent following exposure to both sequences (e.g. 5.4 ± 0.2- vs 5.2 ± 0.2-fold over basal levels; $P > 0.5$). Collectively, these findings suggest that despite their disparate differentiation-inducing abilities, the PKC activators BRY and PMA do not exert qualitatively (or quantitatively) different effects on activation of the caspase cascade in cells previously exposed to ara-C, or on the characteristics of the DNA fragmentation that ensues.

To assess the biologic consequences of these actions, clonogenic survival studies were performed (Table 1). While induction of apoptosis has been shown to reduce clonogenic survival [21], several other studies have demonstrated a discordance between the extent of apoptosis and loss of self-renewal capacity [36, 37]. It can be seen that BRY alone had a relatively modest effect on self-renewal capacity, reducing clonogenicity to 49.1 ± 2.4% of control values, whereas PMA was considerably more inhibitory, reducing colony formation to 7.5 ± 4.7% of control levels. Cells treated with ara-C (1 μM) for 6 hr, washed, and incubated for an additional 24 hr in drug-free medium displayed a very substantial reduction in colony formation (e.g. 98%). In each case, inhibition of clonogenicity was considerably greater than the extent of apoptosis observed at the end of the drug exposure interval. Finally, when ara-C-pretreated cells were exposed subsequently to BRY or PMA, colony formation declined by an additional 75% in each case (e.g. to 0.5 ± 0.2 and 0.4 ± 0.3% of control

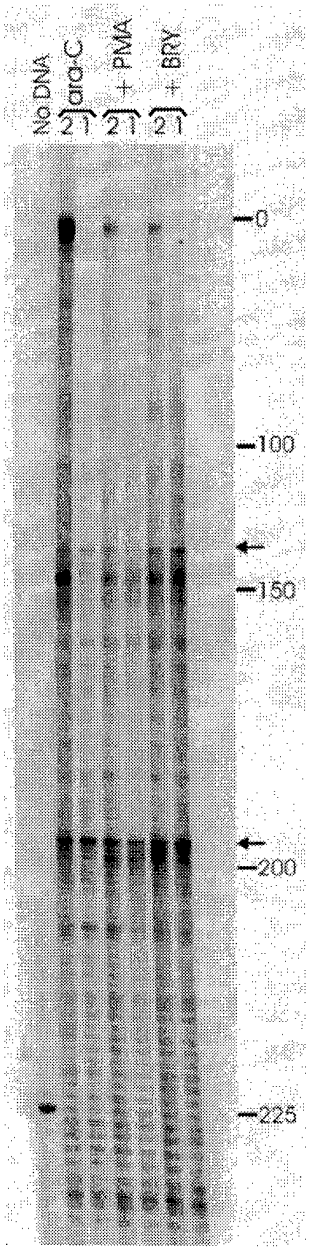


FIG. 3. Cleavage patterns for internucleosomal DNA fragmentation in the *Alu* sequence, as determined by ligation-mediated PCR. Cleavage in the + strand of the *Alu* sequence, for double-strand breaks producing dinucleosomal (lanes "2") and mononucleosomal (lanes "1") fragments, as determined by ligation-mediated PCR is shown. Numbers show *Alu* consensus sequence positions and arrows indicate cleavage hotspots at bases 197–198 (TC ↓ GC) and 142–143 (GG ↓ CG) of the *Alu* sequence, as assigned by reference to restriction enzyme-treated samples.

TABLE 1. Clonogenicity

Treatment	% of Control
BRY	49.1 ± 2.4
PMA	7.5 ± 4.7
ara-C	2.0 ± 0.3
ara-C → BRY	0.5 ± 0.2
ara-C → PMA	0.4 ± 0.3
APC	100.0 ± 9.4
APC → BRY	50.0 ± 8.1
APC → PMA	11.0 ± 1.0

Self-renewal capacity in U937 cells pretreated with either 1 μM ara-C or 1 μM APC (aphidicolin) for 6 hr (4×10^5 cells/mL), washed and further incubated with medium, 10 nM BRY or 10 nM PMA for an additional 24 hr (2×10^5 cells/mL). Values represent the means ± SEM of three separate experiments done in triplicate (N = 9).

values for ara-C/BRY and ara-C/PMA, respectively vs 2.0 ± 0.3% for ara-C alone; $P < 0.001$ in each case). The inhibitory effects of ara-C/BRY and ara-C/PMA did not, however, differ significantly ($P > 0.5$). Thus, while potentiation of ara-C-induced apoptosis by BRY or PMA was accompanied by a further reduction in leukemic cell self-renewal capacity, it appears likely that additional factors contributed to the very extensive loss of clonogenic potential after drug exposure.

Previous reports have shown that BRY and PMA induce expression of the CDKIs $p21^{CIP1}$ and $p27^{KIP1}$ [16, 20, 38, 39], both of which have been shown to influence the apoptotic response of neoplastic cells to various cytotoxic agents [40–42]. To determine what impact prior treatment with ara-C (or dFdC) might have on CDKI induction by PMA and BRY, $p21^{CIP1}$ and $p27^{KIP1}$ expression was monitored in cells exposed sequentially to these agents (Fig. 4). Consistent with the results of other groups [43] as well as our own [24], ara-C treatment alone failed to induce $p21^{CIP1}$ or $p27^{KIP1}$, nor did dFdC increase expression of either of these CDKIs. Unexpectedly, pretreatment of cells with either ara-C or dFdC markedly reduced induction of $p21^{CIP1}$ following a subsequent 24-hr exposure to PMA. A similar phenomenon was noted in the case of $p27^{KIP1}$, i.e. both PMA and BRY induced $p27^{KIP1}$, whereas ara-C and dFdC pretreatment inhibited this response. In view of evidence that interference with the function of CDKIs such as $p21^{CIP1}$ or $p27^{KIP1}$ may increase the susceptibility of malignant cells to drug-induced apoptosis [40, 41], and that enforced expression of $p21^{CIP1}$ may have the opposite effect [42], these findings raise the possibility, although indirectly, that antagonism of CDKI induction by nucleoside analogs may contribute to lethality in cells subsequently exposed to PKC activators.

Given the observation that pretreatment with deoxycytidine analogs opposed CDKI induction (Fig. 4), an attempt was made to determine what impact this action might have on leukemic cell maturation. To this end, cells were exposed to ara-C (1 μM) or dFdC (100 nM) for 6 hr, after which they were washed and incubated in either medium or

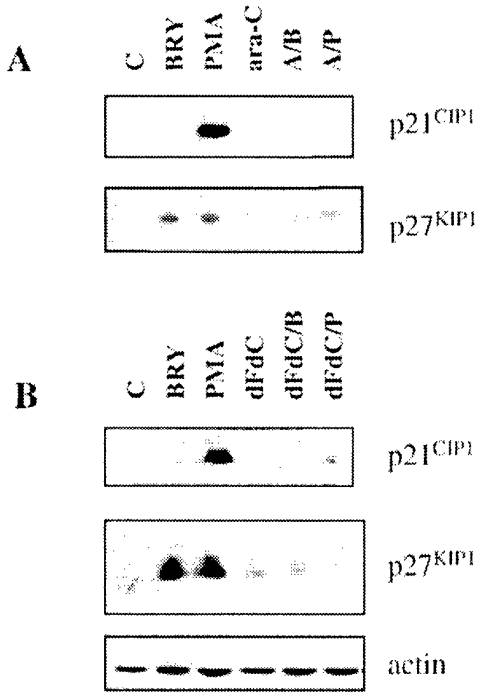


FIG. 4. Inhibition of CDKI expression by PKC activators, BRY or PMA, following pretreatment with dCyd analogs. Cell extracts were pretreated for 6 hr with either (A) ara-C (1 μM) or (B) dFdC (100 nM) followed by an additional 24-hr incubation with 10 nM BRY (B) or 10 nM PMA (P). Equal gel loading was confirmed by amido black staining of the membrane and reprobing with anti-actin antibody.

PMA (10 nM) for an additional 72 hr. At the end of this interval, expression of the myelomonocytic maturation marker CD11b was assayed. Exposure to PMA alone induced CD11b expression in 47% of cells, whereas treatment with ara-C or dFdC alone for 6 hr resulted in CD11b expression in 29.4 ± 3.5 or 20.8 ± 2.7% of cells, respectively (Fig. 5). The latter results are consistent with the established ability of nucleoside analogs such as ara-C to induce leukemic cell differentiation [44]. Interestingly,

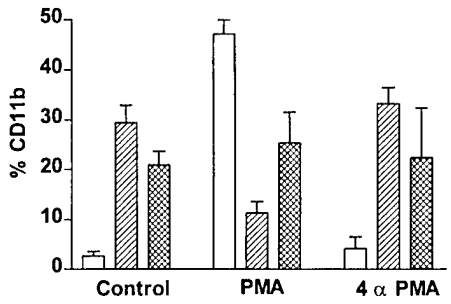


FIG. 5. Inhibition of PMA-induced maturation by pretreatment with dCyd analogs. Shown is CD11b expression in cells pretreated (6 hr) with ara-C (1 μM; right-hatched bars) or dFdC (100 nM; double-hatched bars), washed, and followed by an additional 72-hr incubation with 10 nM PMA or 10 nM 4α-PMA. U937 cells treated with vehicle, PMA or 4α-PMA for 72 hr are indicated by open bars. Values represent the means of triplicate determinations (± SEM).

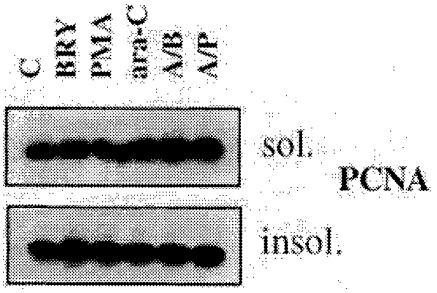


FIG. 6. PCNA distribution after exposure to ara-C. Shown are western blots of cell extracts from U937 cells exposed to ara-C (1 μM) for 6 hr, followed by an additional 24-hr incubation with medium, 10 nM BRY (A/B), or 10 nM PMA (A/P). Detergent-insoluble and -soluble cell fractions are indicated on the right.

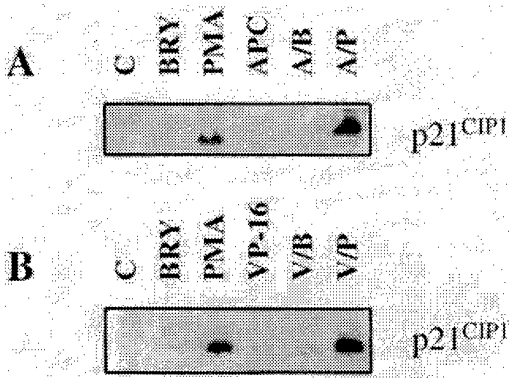


FIG. 7. Effect of pretreatment with APC or etoposide on induction of $p21^{CIP1}$ expression by BRY or PMA. (A) $p21^{CIP1}$ expression in cell extracts pretreated for 6 hr with APC (1 μM) followed by an additional 24-hr incubation with 10 nM BRY (A/B) or 10 nM PMA (A/P). (B) $p21^{CIP1}$ expression in cell extracts pretreated for 6 hr with VP-16 (10 μM) followed by an additional 24-hr incubation with 10 nM BRY (V/B) or 10 nM PMA (V/P).

sequential exposure of cells to ara-C or dFdC followed by PMA exerted clearly antagonistic effects on CD11b expression, resulting in positivity in only 11.4 ± 2.2 and 25.3 ± 6.2% of cells, respectively. In contrast, the inactive phorbol, 4α-PMA, exerted no effect on CD11b expression in drug-pretreated cells (Fig. 5). Results of parallel studies involving the weak differentiation-inducer BRY were less dramatic but also revealed subadditive effects on cell maturation (not shown). These data suggest that one of the consequences of inhibition of PKC activator-mediated CDKI induction by deoxycytidine analogs is interference with the normal maturation program.

$p21^{CIP1}$ is known to form quaternary complexes (also containing cyclins, PCNA, and DNA polymerase δ) [45–47] that are involved in the regulation of DNA synthesis [45, 48, 49]. Although the level of PCNA remains constant in replicating cells, it exists in two separate compartments, i.e. detergent-soluble and detergent-insoluble. The latter represents the functional form bound to DNA at the replication fork [46, 50]. Increases in DNA-bound PCNA have been implicated in potentiation of apoptosis by agents that inappropriately activate CDKs (e.g. UCN-01) [31]. To determine whether a similar event might be associated with ara-C- and dFdC-mediated inhibition of $p21^{CIP1}$ and $p27^{KIP1}$ induction, extracts from cells treated with ara-C followed by BRY or PMA were examined with respect to translocation of PCNA to the DNA-bound, detergent-insoluble fraction (Fig. 6). However, the cellular distribution of PCNA was not discernibly altered by ara-C or dFdC pretreatment, making it unlikely that this mechanism could be invoked to explain the observed effects on apoptosis.

It remained possible that ara-C and dFdC, whose triphosphate derivatives are potent inhibitors of DNA polymerase, might antagonize CDKI induction through a yet-to-be-identified feedback mechanism. To address this possibility, cells were pretreated with 1 μM APC for 6 hr, which, like ara-C and dFdC, inhibits DNA synthesis, but unlike these dCyd analogs, is not incorporated into elongating DNA strands [51]. Treatment with APC minimally induced apoptosis in these cells (e.g. 6.3 ± 0.5%); moreover, sequential exposure of cells to APC followed by either BRY or PMA was not associated with a significant increase in apoptosis (11.4 ± 0.8 and 14.2 ± 2.6%, respectively). Equivalent results were observed using 10 μM APC (not shown). In addition, exposure of cells to BRY or PMA following APC did not lead to enhanced DNA fragmentation (not shown), in contrast to results obtained in cells pretreated with ara-C. Consistent with these findings, APC treatment by itself (1 μM; 6 hr) did not inhibit clonogenicity, nor did pretreatment with APC significantly enhance the inhibitory effects of BRY or PMA (Table 1). Finally, in contrast to ara-C and dFdC, APC failed to block induction of $p21^{CIP1}$ by PMA (Fig. 7A) or $p27^{KIP1}$ by BRY or PMA (not shown). These findings raise the possibility that the observed effects of nucleoside analog pretreatment on apoptosis, loss of clonogenicity, and inhibition of CDKI induction in cells subsequently exposed to PKC activators may be related to analog incorporation into DNA, rather than to inhibition of DNA polymerase *per se*.

To test the possibility that inhibition of CDKI induction by ara-C and dFdC might represent a generalized consequence of drug-induced apoptosis, cells were treated with the topoisomerase II inhibitor VP-16 (5 or 10 μM; 6 hr) followed by a 24-hr exposure to 10 nM BRY or PMA. In contrast to ara-C or dFdC pretreatment, prior treatment with VP-16 failed to block PMA-mediated $p21^{CIP1}$ induction (Fig. 7B). Interestingly, subsequent exposure of VP-16-pretreated cells to BRY or PMA failed to increase apoptosis (i.e. 37.7 ± 1.8% for VP-16 alone vs 35.9 ± 2.6 and 34.8 ± 1.9% for the sequences VP-16 followed by BRY or PMA, respectively; $P > 0.05$). These results argue against the possibility that inhibition of CDKI expression represents a generic response to drug-induced apoptosis. They are also compatible with the hypothesis that interference with CDKI induction contributes to the increase in apoptosis observed when certain cytotoxic drugs are administered prior to PKC activators.

Finally, because induction of $p21^{CIP1}$ and $p27^{KIP1}$ expres-

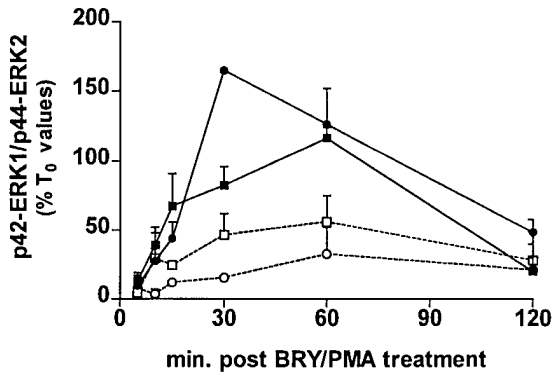


FIG. 8. Inhibition of MAPK activation by ara-C pretreatment. U937 cells were either untreated (control) or pretreated with ara-C (1 μM) for 6 hrs, washed, and further incubated with medium, or 10 nM BRY or PMA for an additional 2 hr. Activity of p42-ERK1/p44-ERK2 was determined by *in vitro* immune complex assay as described in Materials and Methods and expressed as the percent activity of either control or ara-C pretreated cells (time 0 = post-wash). Key: control/BRY (■), control/PMA (●), ara-C/BRY (□), and ara-C/PMA (○). Results represent the means ± range for duplicate experiments.

sion has been shown in response to MAPK [52, 53], the effects of ara-C preincubation on PMA- and BRY-associated activation of MAPK were investigated (Fig. 8). PMA and BRY induced 100–150% increases in basal MAPK activity that were maximal at 30 and 60 min, respectively. Significantly, preincubation of cells with ara-C reduced BRY- or PMA-stimulated increases in MAPK activity. These findings raise the possibility that interference with BRY and PMA-related MAPK activation may be responsible, at least in part, for the observed attenuation of $p21^{CIP1}$ and $p27^{KIP1}$ induction by deoxycytidine analogs.

DISCUSSION

The central goal of this study was to characterize the effects of two PKC activators (PMA and BRY) exhibiting disparate differentiation-inducing capacities in dCyd analog-pretreated leukemic cells. The results described herein indicate that despite the relatively weak maturation-inducing capacity of BRY [17–20], its effects on apoptosis in ara-C- and dFdC-pretreated cells are quantitatively and qualitatively similar to those of the potent differentiation-inducer PMA. Previous studies have shown that induction of leukemic cell maturation is associated with DNA strand breaks [54], and it has been proposed that subsequent differentiation induction may promote drug-induced DNA damage, perhaps by interfering with DNA repair [11]. An alternative possibility is that DNA damage triggered by differentiation-inducing agents, including PKC activators, differs in some fundamental manner from that initiated by cytotoxic agents. However, given the observation that treatment of cells with ara-C alone or ara-C followed by either BRY or PMA resulted in identical DNA cleavage patterns (Fig. 3), it appears that identical endonucleases are involved in each case. Moreover, subsequent exposure of ara-C-treated cells to BRY or PMA resulted in quantitatively (and qualitatively) equivalent effects on caspase-3 activation and degradation of downstream targets (e.g. PARP). Such findings are most compatible with the concept that each of these PKC activators increases the fraction of leukemic cells undergoing drug-induced apoptosis, but does not alter the nature of the cell death program itself. Furthermore, the equivalent effects of BRY and PMA on dCyd analog-mediated apoptosis suggest that differentiation induction *per se* is not primarily responsible for this phenomenon. Instead, they raise the possibility that early, precommitment events that in themselves are insufficient to initiate terminal differentiation account for the observed effects on cell death. Lastly, given evidence that induction of leukemic cell maturation may alter the activity of pyrimidine biosynthetic enzymes (e.g. deoxycytidine kinase; cytidine deaminase) involved in ara-C metabolism [55], it is important to note that neither BRY nor PMA enhanced retention of the lethal ara-C metabolite ara-CTP.

The ability of drugs such as ara-C and dFdC to oppose induction of CDKIs by BRY and PMA has not been described previously, and it seems plausible that this phenomenon may contribute, at least in part, to apoptosis observed in leukemic cells sequentially exposed to these agents. Treatment of leukemic cells with PMA [38, 43] or vitamin D_3 [8] results in induction of $p21^{CIP1}$ or $p27^{KIP1}$, inhibition of cyclin-dependent kinases, and ultimately dephosphorylation of the retinoblastoma protein, inactivation of the transcription factor E2F, and G_1 arrest [56]. The latter process has been shown to be required for normal maturation events to proceed [57]. Furthermore, there is considerable evidence that differentiation and apoptosis represent mutually exclusive cellular fates [7]. For example, U937 cells exhibiting dysregulation of the PKC isoform undergo apoptosis rather than maturation in response to PMA [6]. Conversely, several studies have demonstrated that dysregulation of the CDKIs $p21^{CIP1}$ and $p27^{KIP1}$ promotes drug-induced apoptosis, possibly by interfering with G_1 arrest and leading to uncoupling of S-phase and mitosis [41], or by antagonizing DNA repair [42]. It is tempting, therefore, to speculate that prevention of PKC activator-mediated CDKI induction by pretreatment with ara-C or dFdC contributes to cell death, possibly by disrupting cellular maturation. In this regard, we have reported recently that U937 cells exhibiting dysregulation of $p21^{WAF1/CIP1}$ (through stable expression of an antisense construct) are less susceptible to differentiation induction and correspondingly more susceptible to PKC activator-induced apoptosis, than their control counterparts [58]. In this way, CDKIs such as $p21^{WAF1}$ may direct cells along a differentiation-related pathway and thereby protect them from apoptosis [59]. Thus, the subadditive effects of dCyd analogs and PMA on U937 cell maturation (Fig. 5) and the accompanying potentiation of apoptosis may represent reciprocal consequences of CDKI dysregulation.

The mechanism by which dCyd analogs oppose CDKI

induction remains to be determined. It is has been shown that cell cycle regulation involves complex formation between CDKIs, CDKs, cyclins, and PCNA [45, 46, 48, 49]; moreover, PCNA interacts with DNA polymerase δ to regulate DNA replication and repair [46]. It therefore seemed plausible that inhibition of DNA polymerase by ara-CTP [60] might lead, through an as yet unidentified feedback mechanism, to CDKI down-regulation. However, the inability of APC, which is not incorporated into elongating DNA strands [51], to mimic the actions of ara-C or dFdC argues that nucleoside analog incorporation into DNA rather than inhibition of DNA polymerase, may be responsible for opposing CDKI induction. Furthermore, the failure of VP-16, a potent inducer of apoptosis [61], to exert the same effect suggests that this phenomenon does not merely represent a generalized consequence of cell death. Of possible mechanistic significance is the finding that pretreatment with ara-C or dFdC reduced PMA- or BRY-mediated activation of MAPK, which has been associated with cytoprotective effects [62]. In view of evidence that MAPK induces both $p21^{CIP1}$ and $p27^{KIP1}$ expression [52, 53], it is conceivable that perturbations in MAPK/ERK signaling by dCyd analogs contribute to antagonism of CDKI induction by BRY or PMA. One possibility is that initial activation of MAPK by analogs such as ara-C [63] may in some undetermined way attenuate subsequent activation by PKC activators. It is clear that additional studies will be required to resolve this issue.

It is important to note that while administration of PMA or BRY after ara-C led to a further reduction in U937 cell clonogenic survival, inhibition of colony formation following administration of drugs either individually or in sequence exceeded the extent of apoptosis. Several studies have demonstrated a discordance between apoptosis and clonogenic growth [36, 37], and these have led to speculation that an early commitment to cell death may occur in the absence of classic morphological and biochemical features of apoptosis [37]. Alternatively, inhibition of DNA synthesis (and the accompanying reduction in cell divisions) or induction of non-apoptotic forms of cell death could have the same net effect. In any event, it is unlikely to be coincidental that APC, which failed to (i) inhibit BRY- or PMA-related CDKI induction or (ii) lead to a significant increase in apoptosis in cells subsequently exposed to BRY or PMA, also failed to enhance the inhibitory effects of BRY and PMA on colony formation (Table 1). While these findings do not establish a causal relationship between interference with CDKI induction, promotion of apoptosis, and loss of clonogenicity, they are at least compatible with this concept.

In summary, the present study demonstrates that despite the limited ability of BRY to induce leukemic cell differentiation, it exerts effects that are comparable to those of the potent differentiation-inducer PMA with respect to induction of apoptosis, DNA fragmentation, and activation of the protease cascade in ara-C- and dFdC-pretreated U937 cells. This finding suggests that relatively early precommitment events, unable by themselves to engage a full differentiation program, underlie the observed interaction. Significantly, ara-C and dFdC pretreatment reduced induction of $p27^{KIP1}$ by BRY, and both $p21^{CIP1}$ and $p27^{KIP1}$ by PMA, raising the possibility that dysregulation of CDKIs by drug pretreatment contributes to apoptosis in cells exposed to these agents in sequence. The ability of dCyd analog pretreatment to interfere with PKC activator-mediated CDKI induction may also help to explain the sequence-dependent interactions of these agents on leukemic cell apoptosis [19]. To address this issue further, U937 cell variants stably expressing $p21^{CIP1}$ and $p27^{KIP1}$ antisense constructs have been isolated [58], and studies assessing their responses to the sequential administration of ara-C followed by PKC activators currently are underway. Finally, the present findings may have implication for the rational design of novel antileukemic strategies combining nucleoside analogs with agents acting through the PKC pathway. In this context, Phase I trials of BRY have been completed [64], and plans to combine this agent with ara-C in patients with leukemia currently are being implemented. Importantly, the recent introduction of PMA into clinical trials of humans [65], and its potentially favorable *in vivo* interactions with ara-C [22], suggest a possible role for this agent in the treatment of hematological malignancies. A better understanding of the mechanism(s) governing interactions of such PKC-activators with established antileukemic drugs may lead to more optimized treatment regimens.

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Title: Influence of ionizing radiation on select proteins of the G1 checkpoint and apoptotic pathways: relationship to growth arrest and cell death in breast tumour cells with wild-type p53

Karen J. Magnet and David A. Gewirtz

Departments of Medicine and Pharmacology & Toxicology

Medical College of Virginia

Virginia Commonwealth University

P. O. Box 980230

Richmond, Virginia 23298-0230

USA

Phone: (804) 828-9523

Fax: (804) 828-8079

E-mail: gewirtz@hsc.vcu.edu

kmagnet@hsc.vcu.edu

Running title: Growth arrest and cell death in irradiated breast tumour cells

Key words: DNA damage, p53, cell cycle arrest, apoptosis, MCF-7, ZR-75

Abstract.

Purpose: To determine the capacity of ionising radiation to interfere with cell proliferation and promote apoptotic cell death in two p53-wild-type breast tumour cell lines; to link these effects to molecular components of the p53 DNA-damage response pathway.

Materials and methods: MCF-7 and ZR-75-1 cells received 10 Gy of ionising radiation. Viable cell number was assessed by trypan blue exclusion; cell cycle distribution determined by flow cytometry; apoptosis evaluated by TUNEL analysis; protein expression measured by immunoblotting.

Results: Radiation arrested growth of both MCF-7 and ZR-75-1 cell lines – with cells accumulating in both G_1 and G_2-M – without promoting apoptosis. p53 and $p21^{Waf1/Cip1}$ protein levels increased and Rb was dephosphorylated in both cell lines. E2F1 and Myc decreased in MCF-7 cells, but remained unchanged in ZR-75-1 cells. E2F4 levels did not change in either cell line. Both Bax and Bcl-2 were unchanged in MCF-7 cells; Bax increased by 50% and Bcl-2 decreased 50% in ZR-75-1 cells.

Conclusions: The presence of functional p53, Myc, and E2F1 proteins is not sufficient for radiation-induced apoptosis in the breast tumour cell. Refractoriness to apoptosis may result from preferential activation of the growth arrest arm of the DNA damage response pathway.

1. Introduction

Many cell types that express wild-type p53 undergo apoptosis in response to agents that cause DNA damage. Conversely, the loss of functional p53 is thought to decrease sensitivity to DNA damage (Lowe *et al.* 1993, O'Connor *et al.* 1993, Fan *et al.* 1994). In previous studies (Watson *et al.* 1997), we have compared the effects of ionising radiation (IR) in two breast tumour cell lines: wild-type p53 MCF-7 cells and MDA-MB-231 cells, which express non-functional p53. Irradiated MDA-MB-231 cells were not killed by apoptosis, as might be expected because the lack of functional p53 in these cells would prevent activation of the DNA-damage response pathway. Consistent with results from other laboratories (Zhan *et al.* 1994, Fan *et al.* 1995, Sakakura *et al.* 1996), MCF-7 cells also did not undergo apoptosis in response to IR. Other agents that cause DNA damage likewise do not induce apoptosis in MCF-7 cells (Oberhammer *et al.* 1993, Fornari *et al.* 1994, Fan *et al.* 1995, Fornari *et al.* 1996). Ongoing studies in our laboratory indicate that both MDA-MB-231 and MCF-7 cells fail to undergo apoptosis after exposure to adriamycin (unpublished data). Breast tumour cells, therefore, appear to be resistant to DNA-damage-induced apoptosis, regardless of p53 status (Wosikowski *et al.* 1995, Wagener *et al.* 1996, Watson *et al.* 1997).

MCF-7 cells can, however, be induced to undergo apoptosis when exposed to agents that function via mechanisms other than induction of DNA-damage (Sokolova *et al.* 1995, Toma *et al.* 1997, Eck *et al.* 1998, Gooch *et al.* 1998, Shen *et al.* 1998). This demonstrates that MCF-7 cells do maintain the capacity to undergo apoptosis under certain conditions. The absense of apoptosis after irradiation or exposure to a variety of antitumor drugs, then, suggests a defect in the coupling of DNA-damage pathway activation with induction of apoptosis that may be a characteristic common to many breast tumor cells.

To investigate the influence of irradition on the DNA-damage response pathway, we compared the effects of ionising radiation on cell proliferation in ZR-75-1 and MCF-7 breast tumour cells, both of which express wild-type p53. ZR-75-1 and MCF-7 cells underwent similar growth arrest, without initial cell killing. In spite of the nearly identical growth responses of MCF-7 and ZR-75-1 cells, the expression of select proteins of the DNA-damage response pathway and the apoptotic pathway varied substantially in these two cell lines. Thus, the similar growth arrest and lack of immediate cell killing in response to radiation do not demonstrate a simple or direct correlation with changes in expression of these DNA-damage pathway or apoptotic regulatory proteins.

2. Materials and methods

2.1. *Cell culture*

MCF-7 human breast carcinoma cells were obtained from the NCI-Frederick Cancer Research Facility, and grown in RPMI 1640 medium supplemented with 10% FCS. ZR-75-1 human breast carcinoma cells were obtained from American Type Tissue Culture, and grown in RPMI 1640 medium supplemented with 10% FCS, 4.5 g/L glucose, 10 mM HEPES, and 1 mM sodium pyruvate. Both cell lines were maintained at 37°C in a 5% CO_2 atmosphere.

2.2. *Irradiation*

Cells were plated in T25 flasks at a density of 2 x 10^4 cm^{-2}. The following day, cells were irradiated with a single dose of 10 Gy γ-rays (cesium-137) at a rate of 1.7 Gy/min.

2.2. *Growth inhibition*

Twenty-four to 72 hours after irradiation, cells were released from the flasks by trypsinization and washed once with phosphate-buffered saline (PBS). Viable cell number was determined by diluting cells into 0.4% Trypan Blue Solution, and counting dye-excluding cells in a hemacytometer.

2.3. *Cell cycle analysis and nocodazole treatment*

Cells were harvested by trypsinization at the indicated intervals following irradiation and fixed in 50% ethanol in PBS. Cells were washed and resuspended in PBS, treated with RNase A, and DNA stained with propidium iodide. Cell cycle distribution was determined using a fluorescent-activated cell analyser. Where indicated, nocodazole was added to cells immediately following irradiation, at a final concentration of 150 ng/ml.

2.4 *TUNEL assay*

Cells were harvested by trypsinization and 20000 cells per sample adhered to microscope slides using a Shandon cytospin centrifuge. Cells were fixed with 4% formaldehyde in PBS followed by acetic acid:ethanol (1:2). After washing with PBS, cells were incubated with 1 mg/ml bovine serum albumin in PBS for 30 minutes at room temperature, and washed again with PBS. DNA fragment ends were labelled by incubating the cells with 500 μM fluorescein-12-dUTP and 0.25 units/μl terminal deoxynucleotidyl transferase (TdT; Boehringer Mannheim) in 1X TdT Reaction Buffer [200 mM potassium cacodylate, 25 mM Tris-HCl, 0.25 mg/ml bovine serum albumin, and 2.5 mM cobalt chloride] for 1 hour at 37°C. Cells were washed with PBS and mounted in Vectashield (Vector Laboratories).

2.5. *Immunoblot analysis*

Cell lysate proteins were separated by SDS-polyacrylamide gel electrophoresis and subsequently transferred to nitro-cellulose membranes (NitroBind, Micron Separations). The membranes were incubated in blocking solution [5% non-fat dry milk and 0.05% nonidet P-40 (NP-40) in PBS] for 1 hour at room temperature, then probed with the following antibodies diluted in blocking solution at the concentrations recommended by the manufacturer: anti-p53 (DO-1) and anti-Rb, Pharmingen; anti-p21 (anti-Cip1), Transduction Laboratories; anti-E2F1 (C-20), anti-E2F4 (C-108), anti-Bax (N-20), and anti-Bcl-2 (100), Santa Cruz Biotechnology. To detect Myc protein, culture medium from growing 9E10 hybridoma cells (a generous gift from Dr. John Cleveland, St. Jude Children's Research Hospital) was diluted 1:5 in blocking solution. After incubation with primary antibody, membranes were washed three times in PBS containing 0.05% NP-40. Membranes were then incubated with horseradish peroxidase (HRP) labelled goat-anti-mouse IgG or HRP goat-anti-rabbit IgG antibodies (Kirkegaard and Perry), and antibody

binding detected with SuperSignal Chemiluminescent Substrate (Pierce) and autoradiography.

3. Results

3.1. *Effects of 10 Gy IR on cell proliferation and cell cycle distribution*

Cells exposed to ionising radiation have been shown to die through a variety of mechanisms, including apoptosis, necrosis, and reproductive cell death (Szumiel 1994). The nature of the response, however, may be tissue specific or dependent on alterations in cell-cycle and apoptotic regulatory proteins. This study was designed to ascertain the early response to irradiation in p53-wild-type breast tumour cells.

The growth rate of untreated MCF-7 cells was substantially greater than that of ZR-75-1 cells (figure 1). Nevertheless, exposure of MCF-7 or ZR-75-1 cells to 10 Gy ionising radiation (IR) resulted in essentially identical responses: radiation inhibited cell growth for a period of at least 72 in both cell lines. Although there was a slight decline in cell number between 48 and 72 hours after treatment, viable cell numbers remained essentially identical to initial values. This lack of reduction in the number of viable cells suggests that the primary initial effect of radiation is to inhibit cell proliferation, rather than to induce cell death in these two breast carcinoma lines. Nonetheless, it is well established that radiation at 10 Gy will ultimately compromise clonogenic survival of the breast tumour cell (Fan *et al.* 1995, Nagasawa *et al.* 1995, Sakakura *et al.* 1996).

[Insert figure 1 about here]

An assessment of viable cell number alone is insufficient to conclude that cell death – particularly apoptotic cell death – is not occurring, because there could be an equilibrium between cell growth and cell death, resulting in an essentially constant cell number. Furthermore, apoptotic cells might be counted as viable cells because of their ability to exclude Trypan Blue dye. Therefore, TUNEL analysis was utilised to detect DNA

fragmentation, an indicator of both apoptotic and necrotic cell death. A very small number of apoptotic cells were detected in both untreated and irradiated cells (figure 2). However, radiation did not increase the extent of DNA fragmentation in either cell line.

[Insert figure 2 about here]

To further substantiate the absence of apoptosis and to determine the nature of growth arrest in response to radiation, cells were analysed for cell cycle phase distribution after irradiation (figure 3). Twenty-four hours after treatment with 10 Gy IR, both cell lines showed an increased percentage of cells in G_2-M and a reduction of cells in S phase compared with untreated cells, consistent with the growth arrest presented in figure 1. The percentage of irradiated cells in G_1 was reduced compared with control cells, presumably due to the build-up of cells in G_2-M. Even with this reduction, though, nearly 45% of cells remained in G_1 after irradiation. There are several possible explanations for this: cells arrest in G_1 in addition to G_2-M; arrest in G_2-M is transient, allowing cells to eventually progress to G_1; cells have not had sufficient time to progress out of G_1. To distinguish between these possibilities, cells were incubated with nocodazole, which traps cells in mitosis by disrupting microtubules.

[Insert figure 3 about here]

Nocodazole treatment reduced the percentage of cells in G_1 to 8% for MCF-7 cells and 29% for ZR-75 cells. When cells were exposed to 10 Gy IR in addition to the nocodazole treatment, the percentage of cells in G_1 was increased, comparable to that of irradiated cells without nocodazole. This indicates that cells from the previous cell cycle are not re-entering G_1, and demonstrates a true arrest in G_1. Because the doubling time for ZR-75-1 cells is greater than 24 hours, it is possible that some of the 29% of ZR-75-1 cells that remained in

G_1 with nocodazole treatment had not progressed through the cell cycle to reach G_2-M. In a separate experiment in which cells were analysed 72 hours after treatment with 10 Gy IR without nocodazole, 59% of ZR-75-1 cells remained in G_1 phase, suggesting a true G_1 arrest, rather than insufficient time to progress through the cell cycle (data not shown).

3.2. *Immunoblot analysis of proteins in the DNA-damage pathway*

The similar response of the two p53-wild-type breast cancer cell lines to ionising radiation provided an opportunity to determine whether the propensity of breast tumour cells to undergo growth arrest rather than apoptosis is related to perturbations in specific elements of the DNA-damage response pathway. The effects of 10 Gy IR on the levels of select proteins involved in cell cycle regulation or apoptosis were determined by immunoblot analysis (figure 4).

[Insert figure 4 about here]

As expected, the level of p53 protein in both cell lines increased following irradiation (Kastan *et al.* 1991), doubling within the first two hours, then eventually declining to between 30 to 50% above baseline level by 48 hours (figure 4b). Presumably due to the increase in p53 protein (El-Deiry *et al.* 1993, Dulic *et al.* 1994), $p21^{Waf1/Cip1}$ levels also increased (figure 4c). The increase in $p21^{Waf1/Cip1}$ was much greater for MCF-7 cells than for ZR-75-1 cells (30-fold vs. 3-fold, respectively: note that data for the two cell lines are plotted on separate axes in figure 4c). The final amount of $p21^{Waf1/Cip1}$ protein was similar for the two cell lines, however, because the basal level of $p21^{Waf1/Cip1}$ protein was higher for ZR-75-1 cells than for MCF-7 cells.

An increase in the levels of the $p21^{Waf1/Cip1}$, a cyclin-dependent kinase inhibitor, should result in accumulation of the hypophosphorylated form of the retinoblastoma protein, Rb (Xiong *et al.* 1993). Immunoblot analysis shows that for MCF-7 cells, Rb dephosphorylation was apparent beginning at 2 hours after irradiation, and only the hypophosphorylated form was seen by 48 hours (figure 4a). For ZR-75-1 cells, both the hyperphosphorylated and hypophosphorylated forms of Rb were evident in untreated cells (time=0), and remained relatively unchanged until 48 hours, when the ratio shifted in favour of hypophosphorylated Rb.

The E2F transcription factors are known to modulate the expression of a number of genes involved in cell cycle progression (reviewed in Cobrink 1996). Overexpression of E2F1 promotes apoptosis in co-operation with p53 (Wu and Levine 1994). The expression and activity of E2F proteins are regulated at many levels, including gene transcription, phosporylation, ubiquitin-mediated degradation, and association with hypophosphorylated Rb protein family members (reviewed in Nevins 1998). Here, we determined the effect of ionising radiation on the levels of two of the E2F proteins, E2F1 and E2F4. We were particularly interested in E2F1 because it is the only E2F family member able to induce apoptosis (DeGregori *et al.* 1997), and plays an integral role in the regulation of genes required for S-phase entry (DeGregori *et al.* 1995). For comparison, we assessed E2F4 protein levels because E2F4 mRNA expression remain essentially constant during the cell cycle (Ginsberg *et al.* 1994). As shown in figure 4, d & e, E2F1 and E2F4 protein levels remained constant in ZR-75-1 cells following irradiation. (Note that although the representative immunoblot in figure 4a indicates an apparent decrease in the level of E2F4 at 48 h, cumulative data from three independent experiments, shown in figure 4e, demonstrated no change in E2F4 protein levels.) In contrast, within 24 hours of treatment with 10 Gy IR, E2F1 levels dropped by 50% in MCF-7 cells, while E2F4 protein levels remained unchanged.

One downstream target of E2F1 is c-*myc* (Thalmeier *et al.* 1989). In previous studies we reported that IR reduces expression of c-*myc* in MCF-7 cells (Watson *et al.* 1997). In addition, high levels of Myc protein can induce p53-dependent apoptosis (Hermeking and Eick 1994, Sakamuro *et al.* 1995). In the MCF-7 cells we observed a transient, 30% reduction in Myc protein 4 hours after radiation exposure (figure 4f). The protein levels then increased back to starting levels by 8 hours, followed by a subsequent 50% decrease by 24 hours after irradiation. In contrast, Myc protein levels were not significantly changed in ZR-75-1 cells.

The increase in p53 and $p21^{Waf1/Cip1}$, and the dephosphorylation of Rb, are compatible with the observed growth arrest in G1 in both cell lines. Furthermore, reduced levels of Myc and E2F1 proteins might contribute to the absence of apoptosis in the MCF-7 cells. Conversely, one might expect that the increased levels of E2F1 or Myc proteins, in combination with elevated p53 protein levels, would lead to apoptosis in the ZR-75-1 cells. One possible explanation would be negative regulation of the apoptotic pathway by Bcl-2 family proteins. Indeed, Myc induction of apoptosis can be inhibited by Bcl-2 (Bissonnette *et al.* 1992, Fanidi *et al.* 1992). We therefore measured levels of Bcl-2 protein, as well as levels of the pro-apoptotic protein Bax, in irradiated cells. In MCF-7 cells Bax levels remained constant and Bcl-2 levels were marginally increased, but neither of these changes was significant (figure 4, g & h). In ZR-75-1 cells, Bax protein levels increased by 50%, while Bcl-2 protein levels decreased to 50% of control levels by 48 hours after irradiation.

4. Discussion

Ionising radiation is known to induce apoptotic cell death in a variety of tumour cell lines (Yonish-Rouach *et al.* 1991, Ramqvist *et al.* 1993, Radford *et al.* 1994, Bracey *et al.* 1995). In other tumour cell lines, the absence of an apoptotic response is thought to be a factor conferring reduced sensitivity to radiotherapy or chemotherapy (Lowe *et al.* 1993, Lowe *et al.* 1994). The apoptotic response that occurs after DNA damage is generally associated with the presence of functional p53. The data presented here, however, demonstrate that MCF-7 and ZR-75-1 breast tumour cells, both of which have wild-type p53, fail to undergo apoptosis in response to a relatively high dose (10 Gy) of ionising radiation.

Evidence in support of the failure of irradiation to promote apoptosis in either MCF-7 or ZR-75-1 cells includes the absence of cell death. Viable cell number remains essentially constant over a period of 72 hours following irradiation, with cells accumulating in both the G_1 and G_2-M phases of the cell cycle. Cell cycle analysis fails to indicate the existence of the sub-G_0 population associated with DNA fragmentation. Fluorescence-based in situ DNA fragmentation analysis (TUNEL assay) of irradiated cells confirms the absence of an increase in the DNA breaks that are indicative of apoptosis.

Failure of MCF-7 cells to undergo apoptosis in response to either ionising radiation, the topoisomerase II inhibitors adriamycin and idarubicin, or other modalities that induce DNA damage, has been reported previously from this as well as other laboratories (Zhan *et al.* 1994, Nagasawa *et al.* 1995, Fornari *et al.* 1996, Watson *et al.* 1997, Gewirtz *et al.* 1998). Yet, MCF-7 cells undergo apoptosis in response to a variety of treatment protocols that do not involve damage to DNA (Sokolova *et al.* 1995, Toma *et al.* 1997, Eck *et al.* 1998, Gooch *et al.* 1998, Shen *et al.* 1998). The absence of apoptosis in irradiated MCF-7 cells

could, therefore, be ascribed to alterations in proteins specifically associated with the DNA damage response pathway. One possibility is that the initiation of the G_1 checkpoint arm of this pathway abrogates p53-dependent apoptosis. This hypothesis is supported by data indicating that the apoptotic function of p53 can be dissociated from its transactivation function (Friedlander *et al.* 1996, Hansen and Braithwaite 1996, Ludwig *et al.* 1996) and that p53-mediated transactivation of $p21^{Waf1/Cip1}$ may prohibit apoptosis (Gorospe *et al.* 1997). Furthermore, the dephosphorylation of Rb as well as the decline in absolute levels of E2F1 in irradiated cells would reduce the availability of free E2F1 protein, interfering with the p53/E2F1 component of the apoptotic pathway. Similarly, the reduction in Myc protein levels could interfere with the p53/Myc component of the apoptotic pathway. In terms of select proteins which are thought to be direct effectors of the apoptotic response, the absence of a radiation-induced increase in Bax, coupled with the relatively high levels of Bcl-2, should poise the cellular machinery away from the induction of apoptotic cell death.

Unfortunately, nearly all of these simple explanations for the absence of apoptosis in p53 breast tumour cells suffer under the weight of the data generated from the ZR-75-1 cells, which also have wild-type p53. Although p53 levels are increased in irradiated ZR-75-1 cells, the effects of irradiation on $p21^{Waf1/Cip1}$ levels are but a tenth of the 30-fold increase observed in MCF-7 cells. Similarly, the phosphorylation state of Rb, which is generally linked to the increase in $p21^{Waf1/Cip1}$, is merely shifted towards the hypophosphorylated form in ZR-75-1 cells. The continued presence of the hyperphosphorylated form of the Rb protein, coupled with sustained levels of E2F1, indicates that sufficient free E2F1 should be available to promote apoptosis in concert with p53. Similarly, as Myc levels are unaltered after irradiation in ZR-75-1 cells, the combination of Myc protein and increased p53 should be permissive for p53/Myc dependent apoptosis.

An even stronger argument that radiation should promote apoptosis in irradiated ZR-75-1 cells is based on the increase in Bax levels in combination with decreased Bcl-2 protein. Coupled with the fact that these cells have high levels of the executioner caspase, caspase 3 (Zapata *et al.* 1998), the absence of apoptotic cell death is not explained solely by the radiation-induced alterations in these cell-cycle and apoptotic regulatory proteins. Nonetheless, we are aware that there are multiple regulatory elements associated with the promotion – or prevention – of apoptosis that remain to be investigated.

The question of whether apoptosis is a necessary component of cell sensitivity to radiation remains a matter of considerable controversy (Brown and Wouters 1999). It is well established that irradiated tumour cells may die through other mechanisms, including necrosis and reproductive cell death (reviewed in Szumiel 1994). Nevertheless, the work presented here, coupled with our previous findings in MDA-MB231 cells (Watson *et al.* 1997), supports the premise that breast tumour cells tend to be refractory to apoptosis in response to radiation-induced DNA damage.

Although the current studies do not serve to identify the basis for the absence of apoptosis, recent work in this laboratory indicates that a pathway culminating in apoptotic cell death can be initiated in both MCF-7 and ZR-75- cells exposed to radiation or adriamycin, subsequent to incubation with Vitamin D3 analogues (S. Sundaram and D. A. Gewirtz, manuscript submitted for publication). Consequently, apoptotic cell death in the breast tumour cell in response to DNA damage may require activation of the p53-dependent DNA damage response in concert with an alternative signalling pathway that is permissive for apoptosis.

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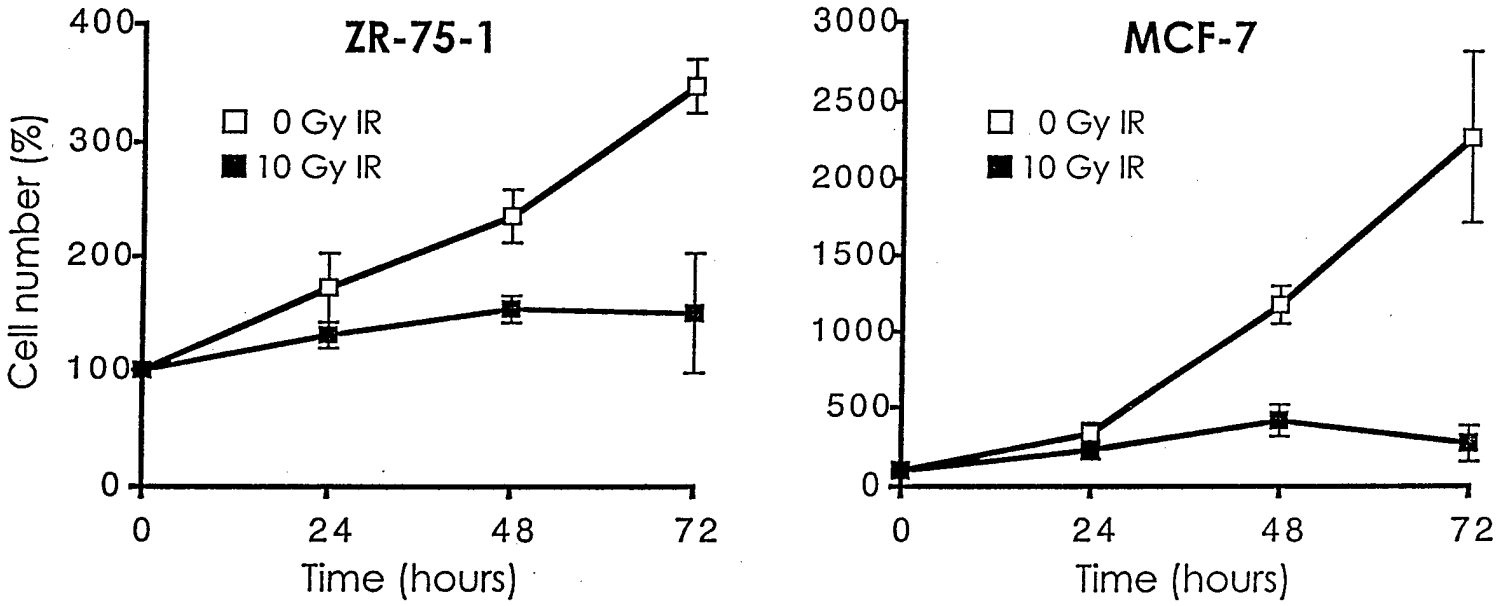
Figure Legends

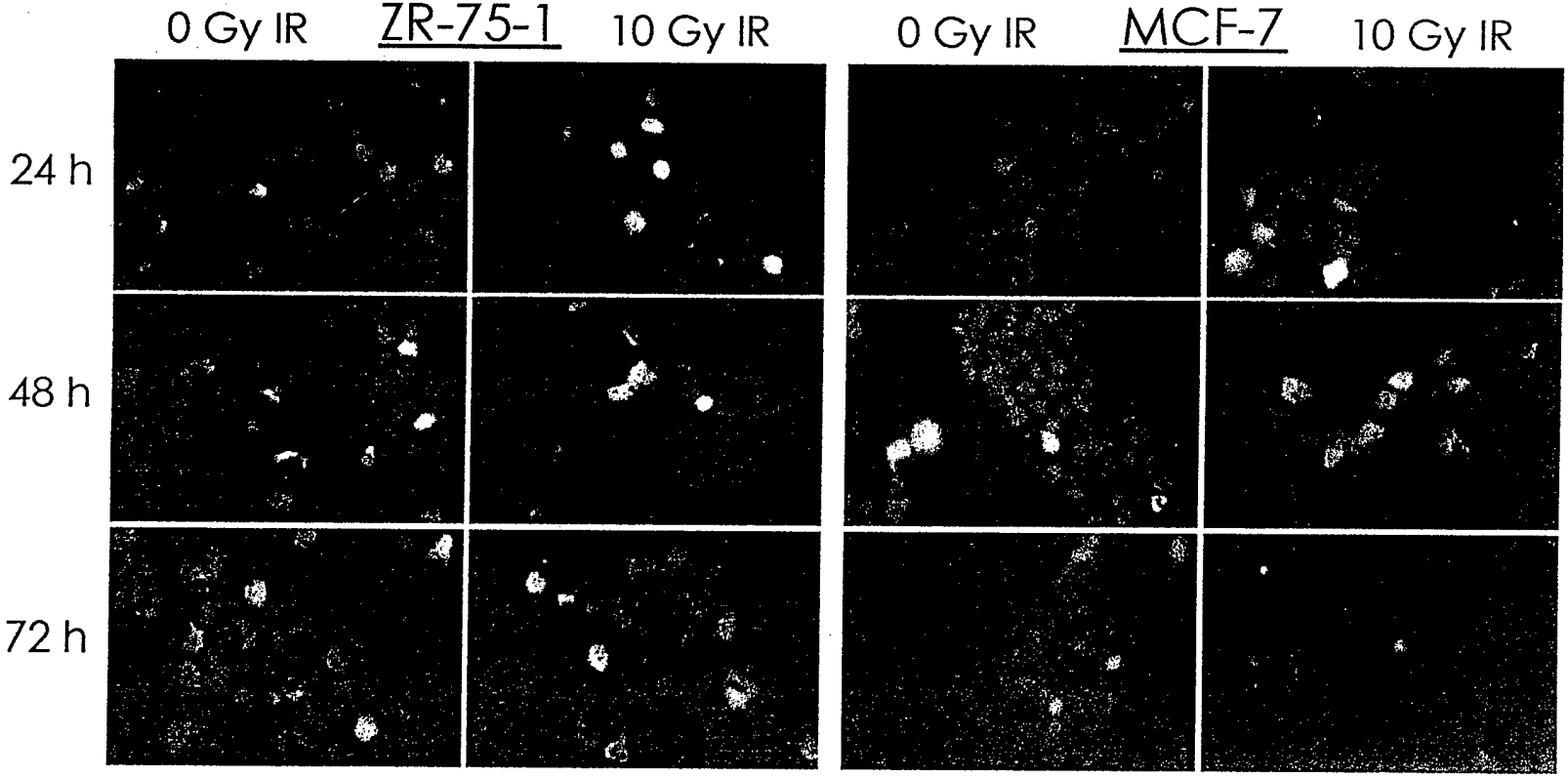
Figure 1. Effect of ionising radiation on cell proliferation. Cells were irradiated with a single dose of 10 Gy; control cells were not exposed to radiation. Viable cell numbers were determined by exclusion of Trypan Blue dye and expressed as percent of original cell number at the time of irradiation. Data shown represent the mean ± standard error of three independent experiments with two parallel cell cultures for each condition and timepoint.

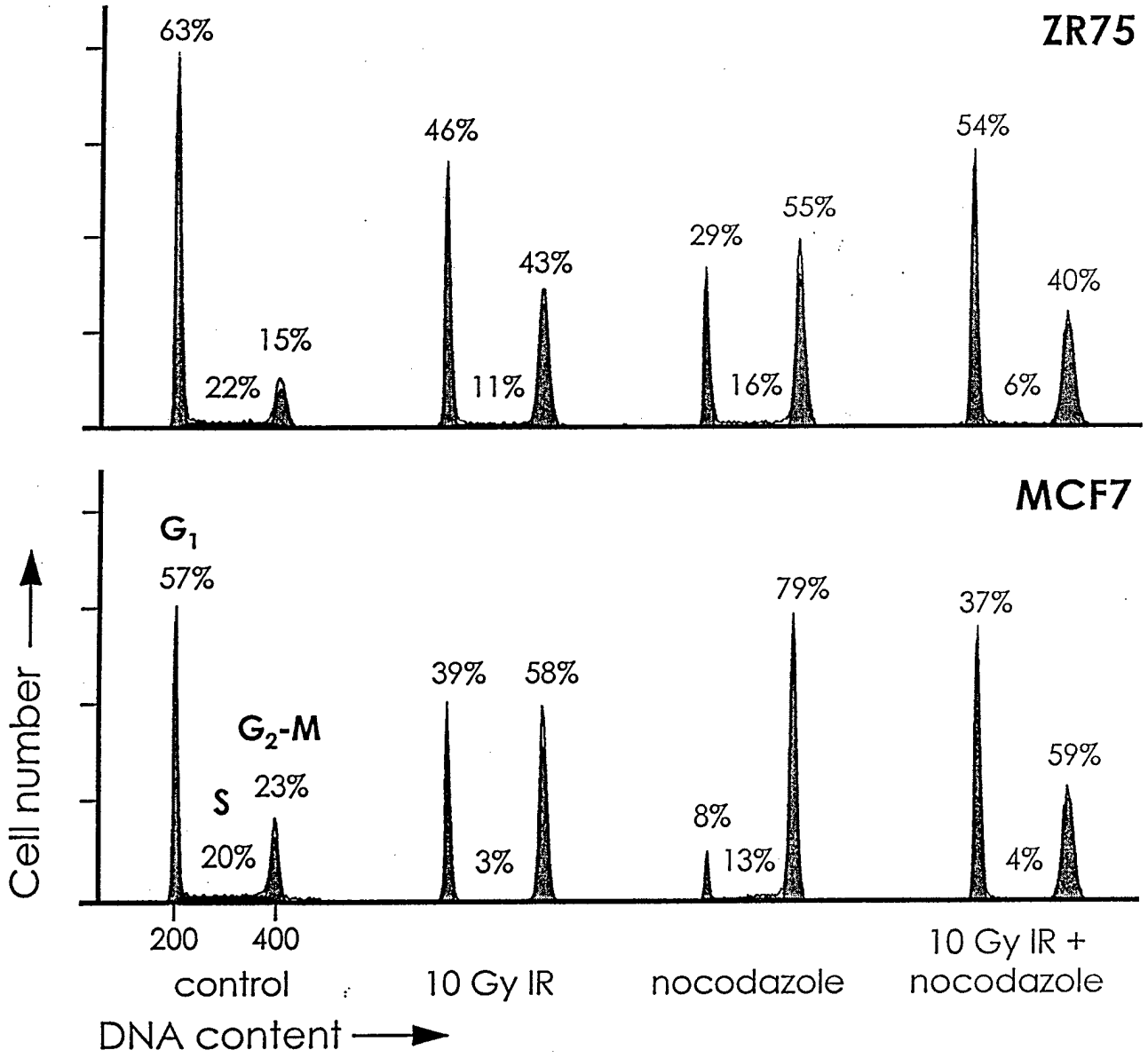
Figure 2. TUNEL assay of irradiated ZR-75-1 and MCF-7 cells. Cells were exposed to 10 Gy of γ-rays; control cells were not exposed to radiation. After the indicated intervals, cells were collected on glass microscope slides and labelled by the TUNEL assay as described in "Materials and methods". Fluorescent cells contain fragmented DNA.

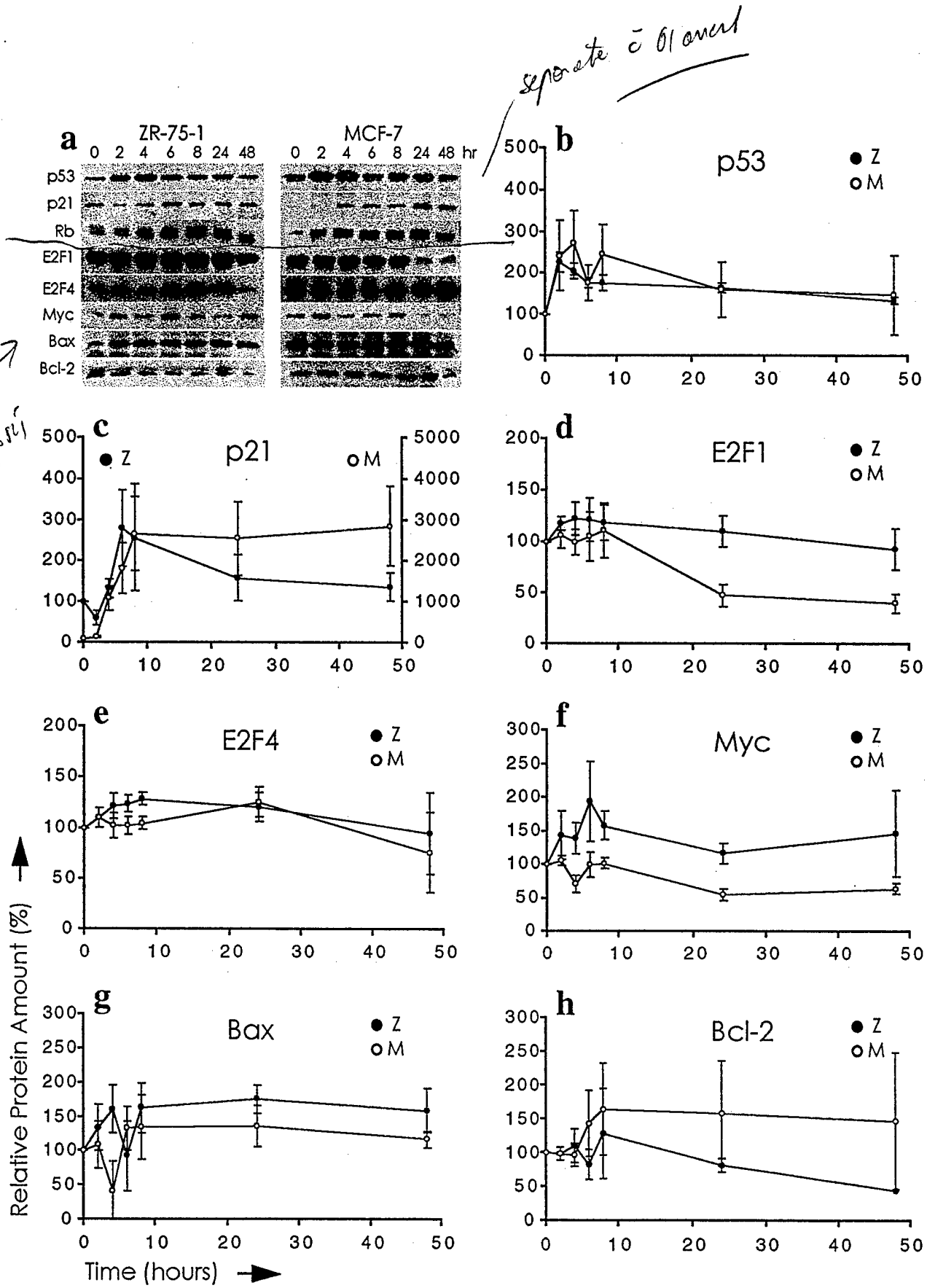
Figure 3. DNA flow cytometry histograms showing cell cycle phase distribution of ZR-75-1 and MCF-7 cells. Cells were analysed 24 hours after exposure to 10 Gy of γ-rays, addition of 150 ng/ml nocodazole, or both. Numbers indicate the percentage of cells in G_1, S, and G_2-M phases.

Figure 4. Time course of protein expression in ZR-75-1 and MCF-7 cells after exposure to 10 Gy ionising radiation. (a) Representative immunoblots of cell proteins probed with the indicated antibodies. (b-h) Quantitative analysis of immunoblots. Data shown represent the mean ± standard error of at least three independent experiments. Note the secondary y-axis in (c): data for ZR-75-1 cells are plotted on the left y-axis; MCF-7 data are on the right.


ZR-75-1
0 Gy IR
10 Gy IR
Cell number (%)
400
300
200
100
0
0
24
48
72
Time (hours)
MCF-7
0 Gy IR
10 Gy IR
3000
2500
2000
1500
1000
500
0
0
24
48
72
Time (hours)


0 Gy IR
ZR-75-1
10 Gy IR
0 Gy IR
MCF-7
10 Gy IR
24 h
48 h
72 h


ZR75
63%
22%
15%
46%
11%
43%
29%
16%
55%
54%
6%
40%
MCF7
G_1
57%
S
20%
G_2-M
23%
39%
3%
58%
8%
13%
79%
37%
4%
59%
Cell number
200
400
control
10 Gy IR
nocodazole
10 Gy IR +
nocodazole
DNA content


a
ZR-75-1
MCF-7
0 2 4 6 8 24 48 hr
p53
p21
Rb
E2F1
E2F4
Myc
Bax
Bcl-2
b
p53
c
p21
d
E2F1
e
E2F4
f
Myc
g
Bax
h
Bcl-2
Z
M
Relative Protein Amount (%)
Time (hours)